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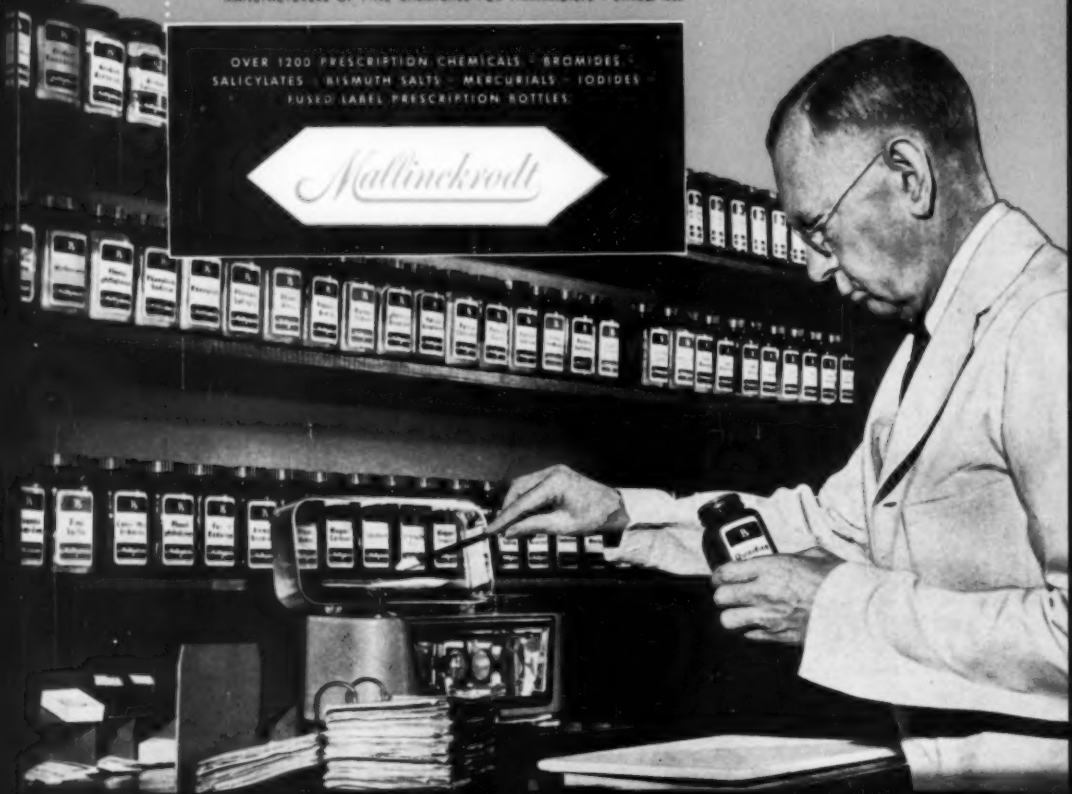
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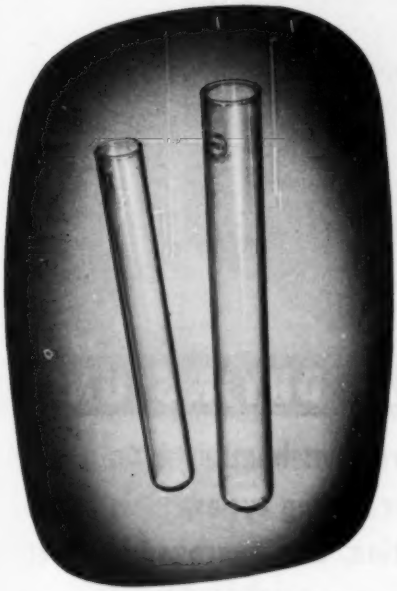

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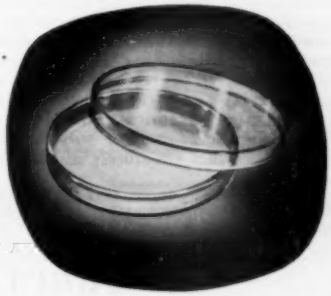

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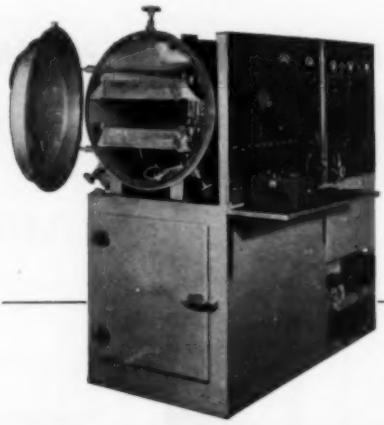

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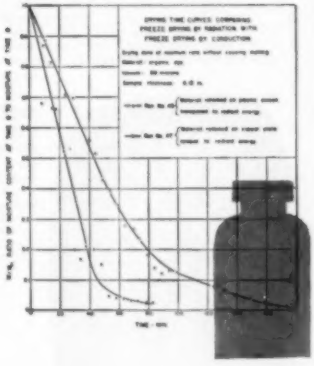

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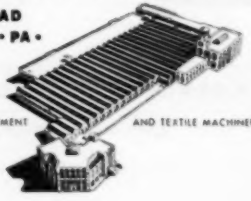

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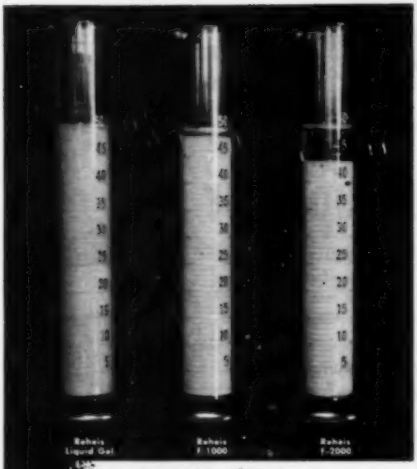

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VOLUME XLI — *NOVEMBER, 1952* — NUMBER 11 — CONSECUTIVE NO. 22

Potentiometric Titration Methods for Antihistamine Bases and Their Salts*

By LEWIS J. KLECKNER and ARTHUR OSOL

When liberated in base form, antihistamines may be dissolved in alcohol and titrated directly with 0.05 *N* aqueous sulfuric acid, or dissolved in a measured volume of the acid and the excess titrated with 0.05 *N* aqueous sodium hydroxide; the end point is determined potentiometrically. A much simpler and more precise method is that of direct titration of either the base or salt form of antihistamines, dissolved in glacial acetic acid, with 0.1 *N* perchloric acid in dioxane; the end point is preferably determined potentiometrically but may also be determined colorimetrically. Interference by the halide acid component of salts is avoided by addition of mercuric acetate.

METHODS for the quantitative determination of antihistamine bases and their salts are almost as numerous as the substances themselves. Gravimetric precipitation as picrate, direct or residual neutralization of base liberated from salts by alkali, Kjeldahl assay for nitrogen, and spectrophotometric determination are the principal quantitative methods in use.

It would be advantageous, at least to those who are concerned with the analysis of a number of antihistaminic substances, to have a general method available. This was the object of the present investigation.

Two methods affording promise of general utility were studied. One of these is a neutralization method which requires initial liberation of antihistamine base, its extraction with ether, and its subsequent estimation by direct or residual neutralization, using aqueous volumetric solutions. The other procedure is that of direct titration of the antihistamine base or its salt, in glacial acetic acid solution, with a volumetric solution of perchloric acid in dioxane.

The underlying principles of the first method are well known. A brief résumé of the fundamental principles of the second method may be noted.

Briefly, this method depends on the fact that when an organic base is dissolved in glacial acetic acid, an equivalent amount of acetate ion is produced, the latter substance acting as a base in the solvent medium. It can be titrated with a solution of perchloric acid, which is a strong acid in the same solvent medium. The net reaction is a combination of acetate and hydrogen ions to form molecular acetic acid which is neutral in the medium. The reaction is analogous to the combination of hydroxyl and hydrogen ions to form water in those neutralization reactions in which water is the solvent medium.

A weak-acid salt of the organic base may be titrated in exactly the same manner, the amount of the organic base determining the amount of acetate (or other basic anion reacting similarly

* Received August 22, 1952, from the Chemistry Department, Philadelphia College of Pharmacy and Science, Philadelphia, Pa.
Presented to the Scientific Section, A. PH. A., Philadelphia meeting, August, 1952.

to acetate ion) which is released. In the case of halide acid salts of organic bases, this procedure will not work directly. Pifer and Wollish (1) discovered, however, that by the simple expedient of adding a solution of mercuric acetate in glacial acetic acid to an acetic acid solution of the halide, an equivalent amount of the acetate of the organic base is formed, along with an equivalent amount of mercuric salt of the halide acid; the latter salt and the excess of mercuric acetate do not react with perchloric acid because of their low degree of ionization.

The use of perchloric acid for titration of bases in a medium of glacial acetic acid has been reported by several investigators; the significant papers have been succinctly summarized by Pifer and Wollish. Confirming an observation of these workers, we have noted that a solution of perchloric acid in dioxane provides a sharper end point than when a solution of perchloric acid in glacial acetic acid is used as the volumetric solution. The end point of the titration may be determined potentiometrically or colorimetrically.

EXPERIMENTAL

Aqueous Volumetric Solution Methods.—The methods employed all utilize the procedure specified in the U. S. P. XIV for extraction of base in the assay of diphenhydramine hydrochloride. In this procedure, a sample of about 300 mg. of antihistamine salt (previously dried at 105° for three hours) is accurately weighed, transferred to a separator, and dissolved in 25 cc. of water. Five grams of sodium chloride is dissolved in this solution, 10 cc. of a 1 in 10 sodium hydroxide solution is added, and the mixture is extracted with 6 or more 15-cc. portions of ether. After washing the combined ether portions with 10 cc. of water, the water washing is extracted with two 10-cc. portions of ether, and the combined ether solution is evaporated in a current of air. At this point a choice of the method of titrating the base must be made. If the base is to be determined by residual titration, 30 cc. of 0.05 *N* sulfuric acid is added (if preferred, this may be added before all the ether is evaporated, after which the remainder of the ether is volatilized by gentle heating), and the excess of acid is titrated with 0.05 *N* sodium hydroxide. The end point is determined potentiometrically, using glass and calomel electrodes.

If direct titration with acid is preferred, the residue remaining after the ether has been volatilized in a current of air is dissolved in alcohol, diluted with an equal volume of water, and titrated with 0.05 *N* sulfuric acid, the endpoint being determined potentiometrically. Correction for acidity of the alcohol is made by potentiometric titration with standard alkali of a portion diluted with an equal volume of water.

The results obtained by these methods, recorded in Table I, agree fairly well with one another. The degree of reproducibility in replicate assays was generally within 0.5%. It would appear that the results may be as much as approximately 1% low in some cases, assuming that the results obtained by titration in glacial acetic acid (described in the following) are correct.

It is noted that in the titration of some of the bases, the change in electromotive force at the equivalence point is rather small (5–10 mv. per 0.1 cc. of volumetric solution), thereby introducing some uncertainty as to the exact position of the end point.

Nonaqueous Volumetric Solution Methods.—The methods employed were those of Pifer and Wollish (*loc. cit.*). A sample of 300–600 mg. (depending on the equivalent weight of the sample), previously dried at 105° for three hours, is accurately weighed and dissolved in 80 cc. of glacial acetic contained in a 250-cc. beaker. If the sample is a halide acid salt, 10 cc. of a 6% (w/v) solution of mercuric acetate in glacial acetic acid is added, and the solution is titrated with 0.1 *N* perchloric acid (prepared by mixing approximately 8.4 cc. of 70–72% perchloric acid with sufficient dioxane to make 1 L.) The end point is determined potentiometrically, using the conventional glass and calomel electrodes for measurement of electromotive force, or colorimetrically, employing 0.5 cc. of a 0.1% solution of crystal violet in glacial acetic acid as the indicator.

The perchloric acid solution is standardized against dried, reagent-grade potassium biphthalate in the same manner as described above.

With the exception of Neohetramine hydrochloride, the potential change at the equivalence point was so great (approximately 40–60 mv. per 0.1 cc. of volumetric solution) that no record of the potential change had to be kept, the equivalence point being readily noted as the point of maximum deflection of the galvanometer needle. With Neohetramine hydrochloride, the potential change was of the order of 15–20 mv. per 0.1 cc. at the equivalence point, the exact determination of which required recording in order to note its position.

The end point may also be determined colorimetrically. As the color change of the indicator varies somewhat with the substance being titrated, the correct color change for each chemical substance is preferably determined, as recommended by Seaman and Allen (2), by observing the color of the indicator at the equivalence point which has been determined potentiometrically. Once the color change is noted, no difficulty is encountered in obtaining results which are both accurate and precise.

In the titration of potassium biphthalate, a precipitate of potassium perchlorate forms in the titration mixture; the same occurs when potassium chloride from the calomel electrode diffuses into the solution being titrated. This precipitate appears to have no influence on the results. Pifer and Wollish reported that calomel electrodes exposed to mercuric acetate in glacial acetic acid are easily contaminated, causing fluctuation of the galvanometer needle and necessitating replacement of the potassium chloride solution inside the electrode. We have been fortunate in not encountering this annoyance.

Confirming an observation of Seaman and Allen (*loc. cit.*), we discontinued use of a magnetic stirrer in the titration beaker when it was observed to

TABLE I.—RESULTS OF ANALYSES OF ANTIHISTAMINES

Sample[a]	Aqueous Volumetric Solution Methods		Perchloric Acid Titration Methods	
	Direct Titration, %	Residual Titration, %	Potentiometric, %	Colorimetric %
Benadryl hydrochloride	99.2	98.7	99.7	99.8
Chlor-Trimeton maleate	99.4	99.0	99.6	99.6
Histadyl hydrochloride	98.9	98.8	99.7	99.9
Neohetramine hydrochloride	98.7	98.6	99.9	100.0
Phenergan hydrochloride	98.5	98.5	99.6	99.5
Pyribenzamine base	98.7	98.1	98.7	98.8
Pyrilamine maleate	98.2	98.4	98.7	98.9
Trimeton maleate	98.5	98.3	99.5	99.4

[a] The authors acknowledge the contribution of supplies of antihistamine bases and salts by Ciba Pharmaceutical Products, Inc., Eli Lilly & Company, Merck & Company, Inc., Nepera Chemical Company, Inc., Parke, Davis & Company, Schering Corporation, and Wyeth, Inc.

cause erratic potential readings in some titrations. Stirring was effected either by swirling the solution manually, or by stirring with a glass rod.

Specially noteworthy is the fact that, while in aqueous solution all the bases (regardless of the number of nitrogen atoms per molecule) reacted with one equivalent of acid, in glacial acetic acid solution Benadryl and Phenergan bases each required one equivalent of perchloric acid; all the others reacted with two equivalents of perchloric acid. The results obtained by titration in the glacial acetic acid medium are also recorded in Table I.

Stability of Perchloric Acid Solution.—Within a day or two after preparation of solutions of perchloric acid in dioxane, a light yellow color develops in the solution if the best commercial grade of the chemical is used (we employed the product manufactured by the Carbon and Carbide Chemicals Corporation). The change in color has no detectable effect on the normality of the solution, even after standing ten weeks. On the other hand, when a "practical" grade of dioxane was used, the solution turned very dark brown within twenty-four hours, rendering colorimetric observation of the end point practically impossible.

Since the temperature coefficient of dioxane is nearly 10 times that of water, it is apparent that temperature changes in the volumetric solution may cause significant errors. As the standardization of the solution, using potassium biphthalate, is very quickly performed, no difficulty should be encountered on this score if the solution is restandardized as required.

The size of drops of a dioxane solution of perchloric acid released by the ordinary 50-cc. burette is half the size of drops of aqueous volumetric solutions; the advantage of this factor in volumetric analyses is apparent.

DISCUSSION

On the basis of experience in the analysis of 8 different antihistaminic substances included in this study, these substances, at least, may be assayed by any one of the methods described in this report. The advantages of titration with the perchloric acid solution clearly indicate the superiority of this method over titrations performed in aqueous or hydroalcoholic solution after preliminary extraction of the base form of antihistamine substances. Only a little more time is required to carry out the titration in glacial acetic acid than to perform a spectrophotometric assay, and the results by the former procedure are both more accurate and more precise. In our opinion, titration in the nonaqueous medium is the most satisfactory method that may be employed for the analysis of organic bases or of salts of these bases.

The gravimetric picrate method is, at least in our experience, a very unsatisfactory general method for the assay of antihistamine substances, principally because of the difficulty of obtaining a satisfactory crystalline precipitate.

As might be expected, salts such as the stearates of calcium, magnesium, or sodium, which are used as lubricants in tablet formulations, interfere by reacting with perchloric acid in acetic acid medium. Pifer and Wollish suggested that when any of these salts is present in a sample containing a halide acid salt of an organic base, the interference due to the former may be avoided by first dissolving the sample in glacial acetic acid, titrating with perchloric acid to neutralize the stearate, then adding mercuric acetate solution to release acetate in an amount equivalent to the halide acid salt, and finally titrating with perchloric acid. This modification will not avoid the interference when an organic acid salt of the organic base is the sample; in this case the extraction procedure utilized in the aqueous neutralization methods should be employed, followed by titration of the organic base in glacial acetic acid solution with perchloric acid.

SUMMARY

1. Eight different antihistaminic substances have been assayed by procedures involving neutralization with aqueous volumetric solutions and with a volumetric solution of perchloric acid in dioxane, respectively.

2. The stability of a solution of perchloric acid in dioxane has been investigated.

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The Effect of *d*-Amphetamine Sulfate on the Level of the Circulating Eosinophils*,†

By CHARLES W. BAUER‡ and MURRAY E. GLICKMAN

A screening study of *d*-amphetamine sulfate was undertaken in the search for new and useful antiarthritic agents. It was shown by standard statistical methods that a significant eosinopenia was produced in the arthritic patient by *d*-amphetamine sulfate. The eosinophil count was used to determine the fall in the level of the circulating eosinophils. The method described suggests a useful pattern to be followed in evaluating other drugs for their effects on the level of the circulating eosinophils, in which no special demands are placed upon the subjects.

As part of the general effort in the search for new and useful antiarthritic agents, a screening study of *d*-amphetamine sulfate was undertaken. This work was suggested and encouraged by Dr. Hans Waine, Medical Director of the New England Chapter of the Arthritic and Rheumatism Foundation, who observed that certain arthritic patients under a regimen of this drug obtained a noticeable degree of relief. To evaluate the drug as an antiarthritic agent, an attempt was made to determine its effect on the level of the circulating eosinophils. A fall in the level of the circulating eosinophils has been shown to be produced by 11–17 oxysteroids as a result of pituitary ACTH administration (1). McDermott, *et al.* (2), reached a similar conclusion, and stated that this eosinopenia is a sensitive measure of adrenal-cortical activity with the secretion of adrenal hormones having an oxygen atom at the C_{11} position. There were several other reasons for the choice of *d*-amphetamine: its chemical relationship to epinephrine and ephedrine; the work of Long (3) and of Vogt (4); the interpretation given by Thorn, *et al.*, of the mechanism of epinephrine stimulation of the anterior pituitary-adrenal cortical system (5); and a parallel screening test made on ephedrine by Abelson and Moyes (6), which showed it to be a good eosinolytic agent.

It was shown by standard statistical methods that a significant eosinopenia was produced in the arthritic patient by *d*-amphetamine sulfate.

The screening tests were carried out on a group of patients at the arthritic clinic of the Boston Dispensary under the supervision of Dr. Heinrich Brugsch, Medical Director. The simple diagnostic tool of the eosinophil count was used to determine the fall in the level of the circulating eosinophils. The full significance of these findings needs further clarification.

EXPERIMENTAL

Although at least a dozen patients were studied in this investigation, seven were finally selected as a sample. Five subjects did not fully cooperate with the program outlined in this study and therefore were not used in evaluating the results. In order to reduce variables to a strict minimum, each subject was first screened by a medical specialist to determine his suitability for such a study. No patients who had recently been under the regimen of other drug therapy could be considered, nor patients who required continued antiarthritic agents. Furthermore, if in the opinion of the medical examiners any attendant medical complication interfered with a freely functioning response, the case was not submitted to the conditions of the experiment.

The pattern of the experiment to study the effects of *d*-amphetamine sulfate[1] was as follows: on the day of the first visit to the clinic, a pre-medication (or control) eosinophil count was taken. The counts were always taken in duplicate, and all were capillary counts from a finger tip. Whenever possible, the count was repeated two or three times or until two out of three counts were approximately the same. Randolph's modification of Thorn's method for the enumeration of eosinophils was used (1, 7). This method proved very satisfactory for the investigation. The use of a propylene glycol diluting fluid aided the stability of the cells in the pipettes whenever it became necessary to transport the specimens from the clinic to the laboratory at the Massachusetts College of Pharmacy. The counts were processed approximately one hour after collection.

Once the patient's control was established, on his next visit he was given a two weeks' supply of Dexedrine with instructions for its administration. The dosage schedule was 10 mg. (two 5-mg. tablets) three times a day before meals, the last dose to be taken not later than 4:00 p. m. The patient was also instructed to begin medication on the morning

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Presented to the Scientific Section, A. Ph. A., Philadelphia meeting, August, 1952.
† This paper is based on a thesis presented to the Graduate Council of the Massachusetts College of Pharmacy by Murray E. Glickman in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.
‡ Professor of Chemistry, Massachusetts College of Pharmacy.

[1] The brand of *d*-amphetamine sulfate used throughout this study was Dexedrine, supplied through the kindness of Smith, Kline and French Laboratories, Philadelphia, Pa.

of the next scheduled visit, so that at clinic time 20 mg. of the drug would have been consumed. At approximately 2:00 p. m. on that day, an eosinophil count was taken for the purpose of observing the immediate effects, if any, of the drug. The time of day for taking the counts was kept constant since the eosinophil count varies with the time of day (8). The eosinophil levels are higher in the afternoon than in the morning. Thus it was felt that any eosinopenia manifested in the afternoon would more truly reflect the activity of the drug. The results recorded in this investigation are based on this fact.

Two double-celled Spencer Brite-Line counting chambers were used. Both sides of each chamber were charged by a separate pipette to make a total volume of 1.8 cm. of diluted blood (1:20) to be examined per count. Since the counts were done in duplicate, 4 cells were examined routinely for each subject. From the experience of Discombe (9) and of Abelson and Moyes (6) the extent of variation among counts was a function of the square root of the number of cells counted. For this reason the procedure was done at least twice on each patient and the average of the two values was taken as the final count. It was also standard procedure to recheck the counts if a discrepancy of more than 2 or 3 cells between the 2 sides of any chamber occurred.

The absolute number of eosinophils was obtained by counting the total number of cells on both sides of a counting chamber; by calculation this value was reduced to represent the level of circulating eosinophils in 1 cmm. of whole blood.

At the very beginning of the work, high power magnification was used, but low power shortly proved to be just as accurate and had the distinct advantage of increased rapidity. The eosinophils were very characteristic and were easily noted with the stain used.

In any screening process, the standard operating procedure cannot be stressed too much. The conditions under which the experiment was performed are very important considerations.

After one, five, eight, and twelve days of medication with *d*-amphetamine sulfate, the group was tested for eosinopenic responses by the method described. From the twelfth to the fifteenth day the drug was withdrawn, and a postmedication eosinophil count was done on the fifteenth day. The object of this step was to determine any return to a normal blood level, provided that any departure from the normal had taken place. This last measurement was made to determine if the postmedication eosinophil level was similar to the premedication level, thus showing more clearly the effects of the medication on the eosinophil count. Furthermore, if the postmedication eosinophil count was of the same order as that of the premedication eosinophil count, it would provide a more reliable control eosinophil count for statistical evaluation.

RESULTS

At the conclusion of the observations, it became necessary to evaluate the data statistically. To test for significance the Student-Fisher "*t*" test was employed, using paired values (10).

The probability for each test was found by consulting the tables of values of Fisher's "*t*," and in each case *P* was found to be approximately 0.02 or less. Therefore, the null hypothesis was rejected, and a definitely significant result was established (see Table I).

Included are graphs of each of the seven cases, which trace the course of progress from the initial premedication control counts through the medication stages to the postmedication period (Fig. 1).

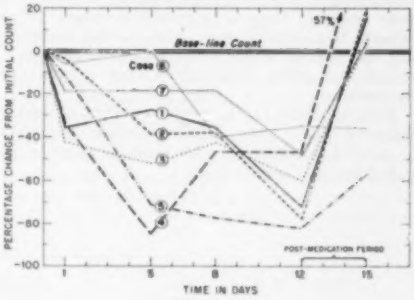

Figure 1.

It can be seen that in all cases an eosinopenia was manifested in the early stages of the experiment and was maintained for its duration, with the greatest drop occurring near the end of the series. In the majority of cases, the postmedication counts returned to or surpassed the initial baseline values. The lowered eosinophil counts ranged from −20 to −60% with an average drop of −42% when all 4 test periods (one, five, eight, and twelve days) were averaged.

That cases VI and VII did not show such sharp decreases during the first eight days of the series (their most marked eosinopenia occurred after twelve days of medication) suggests that the greatest effect of the drug in the dose administered was not reached in the first eight days of medication. The values for the twelve-day medication period range from −35 to −82% with an average of −60%.

Patient VII complained of inability to sleep and lack of appetite; in this case it became necessary

TABLE I.—SUMMARY OF COMPUTATIONS FOR STUDENT "*t*" TEST FOR SIGNIFICANCE

No. Days of Medication	$\sum d_i^2$	$\bar{d}^2$	$\sqrt{\sigma_{\bar{d}}^2}$	t	P
1	5704	497	7.25	3.08	0.02 (approx.)
5	12363	1225	9.5	3.69	0.01 (approx.)
8	14388	1681	7.86	5.22	0.01
12	27862	3364	10.14	5.72	0.01

to eliminate the late afternoon dose and to advise p. c. medication. This change succeeded in enlisting the continued cooperation of the subject. The degree of eosinopenia observed in this case may have been influenced by the change in dosage schedule.

CONCLUSIONS

The maximum effect of *d*-amphetamine sulfate on the level of the circulating eosinophils is less on the first day than it is on the twelfth day. This suggests that there is a cumulative effect of the drug, and that repeated medication is necessary to get the optimum benefit from it.

The method described suggests a useful pattern to be followed in evaluating other drugs for their effects on the level of the circulating eosinophils, in which no special demands are placed upon the subject.

Results of the work suggest that anyone starting an investigation of this type should use at least 50% more subjects than are deemed necessary for a reliable test sample, even after careful screening and selection of subjects, to compensate for absenteeism and other irregularities that are usually encountered.

Pressor amines, as a class, may exert a similar influence on the level of the circulating eosinophils as seen from experience with trials of epinephrine, ephedrine, and amphetamine.

SUMMARY

1. *d*-Amphetamine sulfate therapy causes a significant eosinopenia.

2. *P* was found to be 0.01 for each of the four periods of medication (one-, five-, eight-, and twelve-day periods).

3. The average drop of the eosinophil level for an average of all test periods was 42 per cent.

4. The average drop of the eosinophil level for the twelve-day test period was 60 per cent.

5. The eosinopenia shows a quantitative difference between the immediate and the prolonged effect of the drug.

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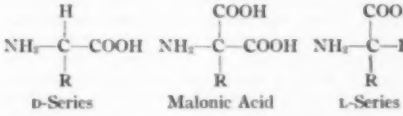
Amino Acids. XI. Malonic Acid Analogs of Natural *alpha*-Amino Acids*

By JAN H. R. BEAUJON and WALTER H. HARTUNG

A study has been made of malonic acid analogs of naturally occurring α-amino acids to determine if stereospecific monodecarboxylation will lead to the formation of optically pure α-amino acids or if these acids possess peculiar biological properties of their own. The synthesis of the malonic acid analogs of leucine, phenylalanine, and tryptophan, via alkylation of carbobenzyloxyaminomalonic ester, is described.

ALPHA-aminoalkylmalonic acids may be considered as α-amino acids with the α-hydrogen atom replaced by a carboxyl group. As such, they offer interesting possibilities. For example, stereospecific monodecarboxylation will give rise to either the D- or the L-series of amino acids, depending on which carboxyl group is eliminated.



Or perhaps they may possess biological properties of their own.

Malonic acids of this type do not appear to be known, although previous workers have used them as unisolated intermediates in the preparation of α-amino acids via the diethyl malonate route. Thus, Hellmann (1), for instance, in his synthesis of DL-tryptophan, assumed the formation of sodium α-aminoskatylmalonate, but he did not isolate or characterize the compound. This is not surprising in view of the ease with which malonic acid derivatives lose carbon dioxide. In the synthesis of α-amino acids from

* Received August 1, 1952, from the School of Pharmacy, University of North Carolina, Chapel Hill.

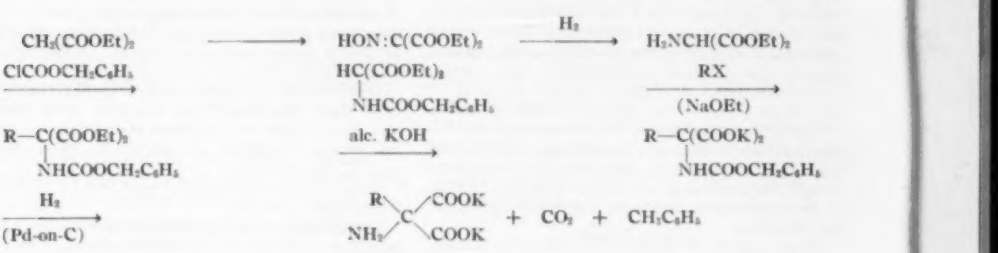
diethyl aminomalonate, the amino function is protected (usually by acylation) before the desired alkyl substituent is introduced; the N-acyl group is then removed simultaneously with hydrolysis of the ester under conditions which are sufficiently strenuous to effect monodecarboxylation of the malonic acid as it forms.

It is now found that diethyl aminomalonate may be converted into its carbobenzyloxy derivative, according to the procedure introduced by Bergmann (2), and the product may be alkylated in the regular manner to form the correspondingly substituted malonic ester of general structure

$$R{-}C(COOC_2H_5)_2.$$
$$\quad\quad|$$
$$\quad NHCOOCH_2C_6H_5$$

From these intermediates it is possible to isolate in good yields, by simple hydrolysis in cold alcoholic acid alkali, either the disodium or the dipotassium salts. leaving untouched the benzyl carbamate grouping. These salts, when subjected to catalytic hydrogenolysis in aqueous solution, give excellent yields of the salts of the desired malonic acid analogs of α-amino acids.

The synthesis may be indicated as follows:



No difficulty was experienced in these reactions; but it should be mentioned that in the hydrogenolysis of the di-alkali salt of carbobenzyl oxyaminobenzylmalonic acid, i. e., in the preparation of the analog of phenylanine, the product was the mono-alkali salt rather than the di-salt, which is not surprising in view of the liberation of one mole of carbon dioxide.

Thus far, we have prepared the malonic acid analogs of leucine, phenylalanine, and tryptophan, selected because the L-amino acids themselves are among those found to be essential (3), and thus will permit a readier comparison of the enzymatic and biological properties of the non-resolvable analogs with the natural substances. There is evidence to encourage the belief that the procedure described may be employed for other members of the aminomalonic acid series.

EXPERIMENTAL

Diethyl Carbobenzyloxyaminomalonate. — The oxime of diethyl mesoxalate, i. e., diethyl oximinomalonate, was prepared according to the method of Cerchez (4) in yields of about 50%,[1] if allowance is made for the recovered diethyl malonate; the oximino compound was reduced to diethyl aminomalonate according to the directions of Barry (5). The amino ester was allowed to react with benzyl chloroformate[2] according to the directions given by Schneider (6), except that sodium hydroxide was used instead of magnesium oxide and extraction was carried out with ether instead of chloroform. Diethyl carbobenzyloxyaminomalonate was isolated as a yellow, viscous liquid, possessing a pleasant odor, as described by Schneider (6). Since the crude product could not be crystallized or distilled without decomposition, it was used without further purification.

Diethyl Carbobenzyloxyaminobenzylmalonate.— In a 500-ml. three-necked flask, equipped with mercury seal stirrer, dropping funnel, and reflux condenser, a solution of sodium ethoxide was prepared from 150 ml. of absolute ethanol and 5.1 Gm. of sodium (0.22 mole), to which was then added 61.9 Gm. of diethyl carbobenzyloxyaminomalonate (0.20 mole if calculated as pure); to the stirred solution was added, over a period of fifteen minutes, 30.4 Gm. of benzyl chloride (0.24 mole). Stirring was continued and the solution was refluxed for four hours, after which the solvent was distilled off; 200 ml. of water was added to the residue in the flask and the mixture was extracted with three 100-ml. portions of ether. The combined extracts were washed with water, the ether was volatilized, and the residue was dried at 35 mm. by heating to 124°, at which temperature evidence of decomposition began to appear. The yield of crude product was 66.6 Gm. (83.5%). The product would not crystallize and could not be distilled.

Potassium Carbobenzyloxyaminobenzylmalonate. —To a filtered solution of 200 Gm. of KOH in 1 L. of 95% ethanol was added 265 Gm. of diethyl carbobenzyloxyaminobenzylmalonate (0.66 mole), which dissolved completely. After about thirty

[1] No other workers have been able to duplicate the yields of 80–90% reported by Cerchez.

[2] Prepared according to the direction of Carter, H. E., Frank, R. L., and Johnston, H. W., *Org. Syntheses*, **23**, 13(1943). When analyzed according to the procedure described, the product was found to contain 0.98 Gm. of benzyl chloroformate per milliliter.

minutes, a white precipitate began to form; after standing overnight, the mixture was filtered, and the precipitate was washed (first with ethanol and then with ether) and recrystallized from ethanol. Yield: 195 Gm. (70.3%).

Anal.—Calcd. for $C_{15}H_{15}O_6NH_2$: N, 3.34. Found: N, 3.32; 3.26.

The corresponding disodium salt, obtained by similar hydrolysis with alcoholic NaOH, was formed with a yield of 90.6%.

Anal.—Calcd. for $C_{15}H_{15}O_6NNa_2$: N, 3.62. Found: N, 3.61; 3.61.

To a solution of 10 Gm. of the dipotassium salt in 100 ml. of water was added concentrated HCl until precipitation was complete. The crystals were suspended in about 1.5 L. of water and the mixture was concentrated by boiling to about 750 ml. After cooling, the crystalline residue was collected, dried, and recrystallized from carbon tetrachloride. A yield was obtained of 2.6 Gm. (33.5%) of carbobenzyloxy-DL-phenylalanine; m. p. 102–103° (uncorr.); reported m. p. 103° (2).

Attempts to prepare pure carbobenzyloxyaminobenzylmalonic acid were unsuccessful. Acidification of an aqueous solution of the salt, although showing no appreciable evolution of carbon dioxide, always gave rise to a crystalline product for which analyses indicated appreciable contamination from carbobenzyloxyphenylalanine.

Potassium Hydrogen α-Aminobenzylmalonate.—A solution of 42.0 Gm. (0.1 mole) of dipotassium carbobenzyloxyaminobenzylmalonate in 100 ml. of water was shaken for three hours in a Parr hydrogenation apparatus with 2 Gm. of Pd-on-C catalyst at a pressure of 4 atm. of hydrogen. There was no appreciable change in the reading on the pressure gauge. The catalyst was removed; the solution was concentrated at reduced pressure to about 40 ml. and then poured into 600 ml. of boiling ethanol. This was filtered while hot, and on cooling, a crystalline product settled out which was recrystallized from 90% ethanol and then dried *in vacuo* over phosphorus pentoxide. A yield of 25.8 Gm. (100%) of monopotassium hydrogen aminobenzylmalonate was obtained.

Anal.—Calcd. for $C_{10}H_{10}O_4NK$: N, 5.66. Found: N, 5.65; 5.72.

By adding one equivalent of KOH to an aqueous solution of the monopotassium salt, the dipotassium salt was formed which could be forced out of solution by the addition of absolute ethanol. On several recrystallizations of the di-salt from ethanol, the monopotassium salt was again obtained.

Anal.—Calcd. for $C_{10}H_9O_4NK_2$: N, 4.91. Found: N, 4.91; 4.94.

Sodium hydrogen aminobenzylmalonate, obtained by similar hydrogenolysis of the corresponding carbobenzyloxy salt, was formed in equally good yields.

Anal.—Calcd. for $C_{10}H_{10}O_4NNa$: N, 6.06. Found: N, 6.12; 6.13.

Like the potassium salt, it was converted to the disodium salt.

Anal.—Calcd. for $C_{10}H_9O_4NNa_2$: N, 5.53. Found: N, 5.76; 5.74.

Diethyl Carbobenzyloxyaminoisobutylmalonate.—From 309.3 Gm. (1.0 mole) of diethyl carbobenzyloxyaminomalonate, 234.0 Gm. (1.4 moles) of isobutyl iodide, and 25.5 Gm. (1.1 moles) of sodium dissolved in 600 ml. of absolute ethanol, a yield of 284.0 Gm. (77.8%) of diethyl carbobenzyloxyaminoisobutylmalonate was obtained. The product did not crystallize and could not be distilled.

Dipotassium Carbobenzyloxyaminoisobutylmalonate.—This compound was prepared from 276 Gm. (0.78 mole) of the ester in the manner described for the benzyl analog. Obtained: 184.5 Gm.; 66.2% yield.

Anal.—Calcd. for $C_{18}H_{17}O_6NK_2$: N, 3.63. Found: N, 3.54; 3.65.

Dipotassium α-Aminoisobutylmalonate.—Dipotassium α-aminoisobutylmalonate was prepared by the hydrogenolysis of 38.6 Gm. of the carbobenzyloxy salt; the yield was 23.0 Gm. (91.3%).

Anal.—Calcd. for $C_7H_{11}O_4NK_2$: N, 5.57. Found: N, 5.56; 5.45.

Diethyl Carbobenzyloxyaminoskatylmalonate.—Diethylcarbobenzyloxyaminoskatylmalonate was prepared from 154.7 Gm. (0.5 mole) of diethyl carbobenzyloxyaminomalonate, 87.1 Gm. (0.5 mole) of gramine, 100 Gm. (0.8 mole) of dimethyl sulfate, and 11.5 Gm. (0.5 mole) of sodium dissolved in 1 L. of absolute ethanol, according to the procedure of Albertson, Archer, and Suter (7) for the preparation of diethyl acetaminoskatylmalonate. Yield: 203.6 Gm. (95.8%) of a very viscous red liquid.

Dipotassium Carbobenzyloxyaminoskatylmalonate.—This compound was prepared from 203.6 Gm. (0.464 mole) of the above ester in the manner previously described. A yield of 174.0 Gm. (81.7%) was obtained.

Anal.—Calcd. for $C_{20}H_{16}O_6N_2K_2$: N, 6.12. Found: 6.03; 6.14.

Dipotassium α-Aminoskatylmalonate.—This compound was formed by hydrogenolysis of 45.9 Gm. (0.1 mole) of the carbobenzyloxy intermediate salt. Yield: 15.0 Gm. (46.2%).

Anal.—Calcd. for $C_{12}H_{10}O_4N_2K_2$: 8.63. Found: N, 8.69; 8.73.

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Amino Acids. XII. Preliminary Nutritional and Enzymatic Studies of Malonic Acid Analogs of Natural *alpha*-Amino Acids*

By JAN H. R. BEAUJON, WILLIAM R. STRAUGHN, JR., and WALTER H. HARTUNG

Four types of simple experiments were carried out with the malonic acid analogs of leucine, phenylalanine, and tryptophan. Results are described.

FOLLOWING the synthesis of the malonic acid analogs of leucine, phenylalanine, and tryptophan (1), four types of simple experiments with these compounds were carried out: chemical decarboxylation, rat feeding, and microbiological and enzymatic studies.

In the chemical monodecarboxylation, the dipotassium salt of α-aminoisobutylmalonic acid was treated with D-tartaric acid, giving a sample of leucine which showed slight optical activity. (+)-Camphorsulfonic acid yielded an optically inactive product.

In the rat feeding experiments, α-aminoskatylmalonic acid was employed. The weanling animals receiving this compound developed alopecia and a severe diarrhea and their survival was not appreciably longer than that of control animals maintained on a tryptophan-deficient diet.

The microbiological experiments with *Lactobacillus arabinosus* 17-5 showed that the growth produced by α-aminoskatylmalonic acid in a concentration of 50 μg./ml. was about equal to the growth produced by 1 μg./ml. of L-tryptophan. It is not yet known whether the malonic acid analog possesses one fiftieth the activity of the natural amino acid, or whether the organisms may have effected partial sterospecific monodecarboxylation, or whether tryptophan may have been present as an impurity in the malonic acid compound formed during the synthesis. Additional preliminary experiments with the analogs of tryptophan, phenylalanine, and leucine show that none has any antimetabolic action on this organism.

In the enzyme experiments the anilides and phenylhydrazides of L-leucine (2) and L-phenylalanine (3-5) were obtained from α-aminoisobutylmalonic acid and α-aminobenzylmalonic acid respectively, in the presence of aniline or phenylhydrazine by means of papain or ficine. Because the yields in all cases were below 50 per cent of theory, it is not clear whether decarboxylation to a racemic mixture preceded resolution or whether there was stereospecific formation of the hemiamides followed by monodecarboxylation. The results with ficin indicate greater specificity than those with papain.

In the present experiments, the potassium salts of the malonic acids were employed because of their greater stability. These studies are being continued.

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Some Physical Properties of Rutin*

By C. F. KREWSON and J. NAGHSKI†

Approximate solubility of rutin in common solvents and solvent-water mixtures has been determined at room temperature and near boiling temperatures. Data are presented showing the formation of rutin solvent complexes. Rutin of high purity, free of quercetin and other flavonoids, has been prepared using this principle. Values for the optical rotation of rutin in various solvents and water-solvent mixtures are given.

SINCE the first report by Griffith, Couch, and Lindauer (1) that rutin is effective in the treatment of spontaneous increased capillary fragility in humans, numerous articles have appeared on its pharmacology and clinical applications. These have recently been summarized (2). Although rutin has been known for over a century, very little has been published on its physical properties. Some of its chemical properties have been brought to light in the elucidation of its structure (3, 4).

Rutin has been isolated and prepared in the pure state from over sixty species of plants. However, the techniques used did not lend themselves to large-scale production of a pure substance for medicinal use. During our development of a successful method for the extraction and purification of rutin from domestic plants (9) it became necessary to acquire considerable data concerning its physical properties. There was a special need for information concerning the approximate solubilities of rutin in water, common organic solvents, and water-solvent mixtures in order to develop optimum conditions for extraction and purification. This paper is a report of some of the studies made on the solubility of rutin in various solvents, its purification, the formation of solvates of crystallization with organic solvents, and its optical rotation in various solvents.

EXPERIMENTAL

Approximate Solubility of Rutin in Various Solvents

Procedure.—An excess of finely powdered rutin of N. F. IX (5) quality, dried eighteen hours at 110° [not anhydrous (10)], was added to 100 cc. of solvent (technical grade) and refluxed for ten minutes. The undissolved rutin was removed by filtering the mixture while hot through a sintered glass disc, using applied pressure to prevent concentration of the filtrate by evaporation.

The amount of rutin dissolved was determined by evaporating the solvent from an aliquot, moistening the residue with water to remove traces of solvent, and finally drying at 110°. Although this procedure does not give the solubility at the exact boiling temperature, it does give a value sufficiently close to the boiling point to be of practical use. To determine the solubility at room temperature, the remainder of the filtrate was placed in a stoppered flask and left at 25–27° for three days. All samples from which rutin precipitated on standing were filtered, and the rutin remaining in solution was determined by evaporating an aliquot to dryness as described above. The data obtained for various solutions of methanol, ethanol, isopropanol, and acetone with water are plotted in Figs. 1–4.

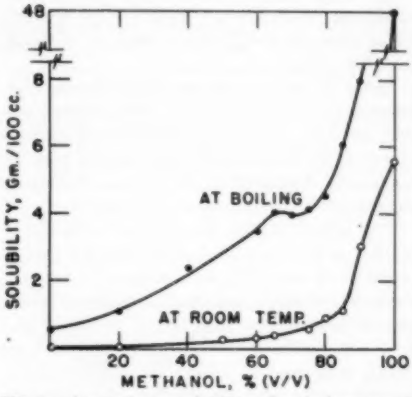

Fig. 1.—Approximate solubility of rutin in aqueous methanol.

Solubility of rutin in water below boiling was determined by maintaining the suspension of rutin in a water bath with stirring for four to six hours. Approximate values for solubility at elevated temperatures were obtained by treating under increased pressures as follows:

The rutin was suspended in water in a sintered glass Büchner funnel which was fitted to an Erlenmeyer flask. This was heated in a steam autoclave for seventy-five minutes. During the heating, steam displaced the air in the flask which, in escaping through the sintered glass, agitated the rutin solution. When the autoclave was turned off, condensation of the steam in the flask created a vacuum and automatically filtered the rutin solution at the elevated temperature. Data obtained

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at various temperatures are plotted on the curve shown in Fig. 5.

Discussion.—The solubility curves (Figs. 1–4) with the organic solvents near the boiling point showed two maxima. One occurred in the anhydrous solvent and the other in water solutions containing 50–70% solvent. The minimum solubility was with solvents of 70 to 98% concentration. Of the four solvents compared, rutin was most soluble in anhydrous methanol, and was soluble in decreasing amounts in strong ethanol, isopropanol, and acetone. Rutin did not precipitate from solutions of ethanol and acetone in the range of the minimum solubility when cooled to room temperature. Thus, values could not be obtained by this procedure for the solubility of rutin at room temperature in these solvents at these concentrations. This is illustrated by the breaks in the curves shown in Figs. 2 and 4. Also, rutin did not precipitate from strong isopropanol (98–99%) solutions, and the break in the curve occurred near the maximum solubility (Fig. 3). Rutin precipitated from solutions of 96–99% ethanol only when seeded with rutin ethanolate (6). However, rutin did not precipitate from a 95% ethanol solution even after seeding with either rutin ethanolate or rutin hydrate. For practical purposes, rutin is insoluble at room temperature in aqueous solutions containing less than 30% solvent.

It is to be noted that the solubility of rutin in water was influenced very greatly by the temperature, especially near the boiling point. Elevating the temperature by raising the pressure to 15 p. s. i. increased the solubility threefold.

Some additional data have been obtained on the solubility of rutin in other solvents. Rutin was

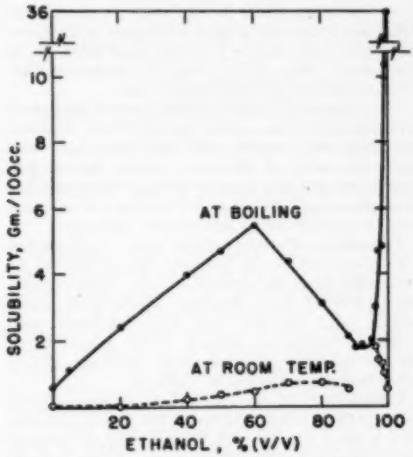

Fig. 2.—Approximate solubility of rutin in aqueous ethanol.

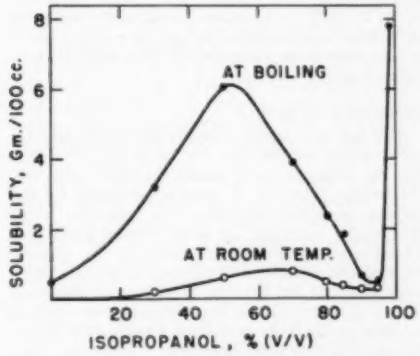

Fig. 3.—Approximate solubility of rutin in aqueous isopropanol.

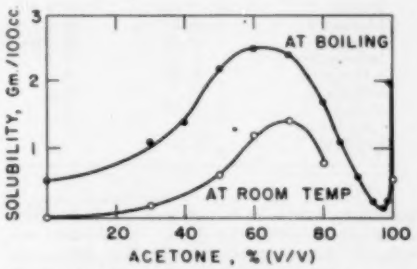

Fig. 4.—Approximate solubility of rutin in acetone.

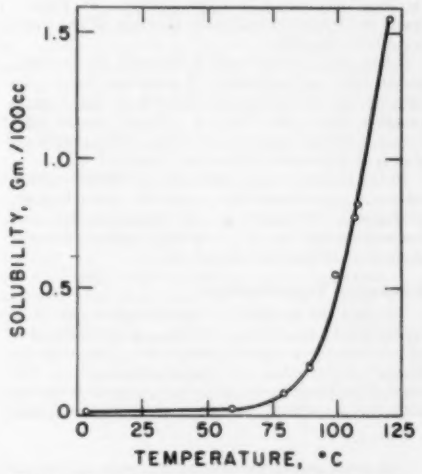

Fig. 5.—Approximate solubility of rutin in water.

TABLE I.—APPROXIMATE SOLUBILITIES OF RUTIN IN WATER AND VARIOUS ANHYDROUS SOLVENTS AT ROOM TEMPERATURE AND NEAR THE BOILING POINT

Solvent	Sol. at Room Temp., Gm./100 cc.	Sol. near Boiling Temp., Gm./100 cc.
Water	0.013	0.50–0.55
Methanol	5.5	48.0
Ethanol	0.55	36.0
Isopropanol	1.45	7.0
n-Propanol	0.60	4.0
n-Butanol	...	70.0
Acetone	0.56	2.0
1,4-Dioxane	...	13.6
Pyridine	37.3	...

exceedingly soluble in hot glycerol (U. S. P., 98% strength) and in ethylene glycol. No attempt was made to dissolve more than 50 Gm./100 cc. of glycerol because of difficulties in obtaining uniform heating. Rutin crystallized readily from diluted glycerol solutions on cooling. It was soluble to the extent of 1.2, 1.4, 1.7, 3.8, and 14.7 Gm./100 cc. in 15, 20, 25, 40, and 72% (v/v) boiling aqueous glycerol solutions, respectively. Boiling *n*-butanol (technical) dissolved 70 Gm. of the rutin used per 100 cc. (The solution gelled on cooling; *anhydrous* rutin may be even more soluble.) The presence of water in the *n*-butanol greatly depressed the solubility. This solvent, to which 5% water was added, dissolved only 0.4 Gm./100 cc., while *n*-butanol saturated with water dissolved 0.7 Gm./100 cc. Boiling absolute *n*-propanol dissolved 4.0 Gm./100 cc., but this alcohol dissolved only 0.6 Gm./100 cc. at room temperature. Boiling 1,4-dioxane (technical) dissolved 13.6 Gm./100 cc. In Table I are listed solubilities of rutin in some of the more important solvents.

Rutin was soluble in such solvents as pyridine, acetic acid, and solutions of inorganic bases. In pyridine it was extremely soluble at room temperature, 37.3 Gm./100 cc. Glacial acetic acid behaved toward rutin in a manner which suggested that it also formed a solvate with rutin.[1]

Rutin has been found insoluble in ether, benzene, toluene, petroleum-ether, carbon tetrachloride, chloroform, chlorobenzene, ethylene dichloride, and 1-nitropropane; it was slightly soluble in butyl acetate at boiling temperatures.

Solvents of Crystallization

No data are available in the literature for rutin on the existence of other solvents of crystallization in addition to water of hydration. This may be because rutin, when not highly purified, does not crystallize from ethanol with ease unless it is seeded with the alcoholate, and then only from solutions containing more than 96% ethanol by volume. During the course of removing alcohol insolubles (7–9) from rutin, an alcoholic solution of rutin suddenly precipitated crystals and quickly set to a solid mass. The product so formed proved to be rutin alcoholate, i. e., rutin with ethanol of crystallization. This product when isolated dissolved easily and abundantly in cold water, a spectacular difference from rutin's normal behavior with water. However, rutin persisted in this solubilized condition only a short time; then the water-insoluble hydrate formed and precipitated out.

Investigation of other solvents showed that rutin crystallized in the same manner from absolute methanol, from acetone, and from *n*-propanol with increasing order of difficulty. Rutin formed gels with *n*-butanol and isoamyl alcohol which did not crystallize: no precipitation of rutin has occurred from strong (98–99%) isopropanol solutions.

Procedure.—Purified rutin of N. F. IX (5) quality (10.0 Gm./100 cc. of solvent) was permitted to crystallize from the desired solvent. The crystals were collected on a Büchner funnel with gentle suction, and the wet cake was distributed into weighing bottles for drying. The quantities of various solvents retained under different drying conditions are presented in Table II.

Discussion.—It was difficult to remove organic solvents from rutin. Although the values obtained do not agree exactly with theory, they do indicate the existence of rutin as trisolvate. Rutin hydrate is practically tasteless, but the alcoholate is extremely bitter. This is in line with expectation for the taste of a glycosidic compound. The contrast is undoubtedly due to the extreme difference in solubility in cold water between the hydrate and alcoholate.

The effect of solvent strength on the preparation of rutin ethanolate was investigated in some detail. Aqueous solutions containing 96% (v/v) or more of ethanol yielded rutin ethanolate while those below 90% ethanol yielded the hydrate. Solutions of 90 to 95% (v/v) ethanol saturated with rutin at boiling temperatures did not precipitate rutin in any form even when seeded with ethanolate or hydrate at 5°.

Purification of Rutin

The fact just reported that rutin forms solvates with various organic solvents led to a new method for the preparation of highly purified rutin (6). Buckwheat rutin (N. F. quality) prepared by the present accepted procedure (5) contains small amounts of quercetin and other related flavonoids. These minor flavonoids interfered in the spectrophotometric analysis, and values greater than 100% were obtained for the sum of rutin and quercetin. Repeated recrystallizations from water or aqueous solvents as the hydrate did not significantly reduce the quantities of these impurities. However, when rutin crystallized as the solvate, the major portion of the impurities remained in the organic solvent solution. The separation and identification of these minor flavonoids will be the subject of a later paper.

The effect on the purity of rutin when samples were crystallized from technical absolute methanol, ethanol, *n*-propanol, and acetone is shown in Table III.

[1] When glacial acetic acid was added to 2.000 Gm. of rutin (moisture, 7.7%) burette-wise with heating under reflux, 15 cc. of the solvent completely dissolved the rutin at boiling temperature. Immediately after the rutin dissolved, a precipitate formed. The quantity of this precipitate increased with the addition of more solvent. Finally 44.1 cc. of acid was required to completely dissolve this precipitate; solubility of rutin on this basis was 4.2 Gm./100 cc. However, in a repeat trial where 20 cc. of glacial acetic acid was run in all at once and the solution was boiled, the rutin dissolved completely. Addition of more acetic acid did not cause any precipitation nor did a precipitate form on cooling; approximate solubility on this basis, 9.2 Gm./100 cc.

TABLE II.—REMOVAL OF ORGANIC SOLVENTS FROM RUTIN BY DRYING

	Drying Conditions	Solvent Retained by Solvated Rutin %	Moles/Mole
Ethanol[a]	Desiccator, $CaCl_2$, room temp., 2 mo.	17.1	2.7
	Desiccator, H_2SO_4, room temp., 2 mo.	6.9	1.0
	Vacuum oven, 65°, 48 hr.	6.3	0.9
	Mech. conv. oven, 110°, 48 hr.	3.6	0.5
Methanol[b]	Vacuum desiccator, $CaCl_2$, room temp., 10 days	14.0	3.1
	Vacuum desiccator, H_2SO_4, room temp., 10 days	1.6	0.3
	Vacuum oven, 65°, 48 hr.	0.4	0.1
Acetone[c]	Acetone atmosphere, 3 days	31.0	3.6
	Vacuum, room temp., 3 days	6.2	0.7
	Vacuum, room temp., 8 days	4.6	0.5
	Vacuum, room temp., 15 days	4.2	0.5
	Vacuum, room temp., 22 days	3.9	0.5

[a] Ethanol content determined, using the technique of Zeisel (15).
[b] Methanol by difference after determination of rutin, using spectrophotometric method (11).
[c] Acetone determined by method of W. M. Marriott (16).

Procedure.—The procedures used for these purifications are described below:

Rutin Methanolate.—Ten grams of rutin dried at 110° was dissolved by heating in 50 cc. of absolute methanol and filtered through a heavy filter pad.[3] The pad was washed with 10 cc. of solvent. Crystallization took place immediately on cooling. Crystals were filtered off after two hours of standing at room temperature; they were slurried with solvent and filtered with suction, and the filter cake was added to 400 cc. of boiling water. The product dissolved immediately, then quickly precipitated as the rutin hydrate. The latter was recovered by filtration after standing sixteen hours and dried at 110°; yield was 5.2 Gm. (52%). Upon evaporation, the mother liquor and washings yielded 4.1 Gm. of additional rutin.

Rutin Ethanolate.—Technique was repeated as described above for methanol except that 5.0 Gm. of dried rutin and absolute ethanol was used. Crystallization occurred immediately when the cooled solution was seeded with rutin ethanolate. Also, the rutin ethanolate cake was placed in 100 cc. of boiling water for hydrate formation. The yield of purified rutin was 3.7 Gm. (74%).

This procedure was applied successfully to the purification of more than 20 kilogram-batches. Yields ranged from 745 to 924 Gm. Concentration of the mother liquors yielded from 50 to 200 Gm. of additional rutin of inferior quality to the main crop.

Rutin n-Propanolate.—The technique was the same as that described for methanol except that only 2 Gm. of rutin was used and crystallization occurred after 7 days of standing at room temperature. In the hydrate preparation step, 150 cc. of boiling water was used; yield was 1.6 Gm. (80%). However, this was still impure as shown by data in Table III.

Rutin Acetonate.—Five grams of dried rutin was dissolved in 300 cc. of acetone (99.5%) and filtered. The product crystallized on cooling in the form of extremely long thin needles. The hydrate was prepared by dissolving the acetonate thus formed in 150 cc. of boiling water in which the acetonate was immediately soluble; yield of rutin was 3.5 Gm. (70%).

Discussion.—Of the four solvents used in purification of rutin, ethanol and acetone gave the purest products. The yields were good since the second crop of rutin obtained from mother liquors may be reprocessed. Ethanol is the most practical of these two to use because the solubility of rutin is greater in ethanol than in acetone and the amount of moisture present in the solvent is not as critical. The solubility of rutin in absolute ethanol is greater than 10%, but more concentrated solutions were difficult to handle especially after the laboratory became seeded with alcoholate. When the laboratory did become seeded, the alcoholate precipitated from absolute alcohol containing more than 10% rutin, even while the solution was boiling under reflux.

Purification of rutin by alcoholate procedure is now in operation on a commercial scale for the production of highly purified rutin required to make soluble preparations of rutin for parenteral use. Rutin prepared through the alcoholate procedure was the only rutin we have obtained which did not discolor when exposed to light; rutin recrystallized 15 times from aqueous solvents became discolored.

Optical Rotation of Rutin

Buckwheat rutin of N. F. IX (5) quality was specially purified for this work by three recrystallizations through the alcoholate procedure as described in the previous section. A determination of its purity was made by spectrophotometric analysis (11) and found to be 100.1% rutin and 0.0% quercetin. Since anhydrous rutin cannot be weighed accurately the sample was equilibrated with air-moisture (a moisture determination showed 7.7%). The solvents used for dissolving the rutin were C. P. reagents. Pyridine was further purified by twice distilling, using only center fractions; the

TABLE III.—EFFECT OF RECRYSTALLIZATION FROM ORGANIC SOLVENTS ON PURITY OF RUTIN[a]

Recrystallized From	Spectrophotometric Analysis Rutin, %	Quercetin, %
Methanol	98.3	1.4
Ethanol	100.0	0.0
n-Propanol	100.6	1.3
Acetone	100.5	0.0

[a] Original rutin analyzed 97.3% rutin and 4.3% quercetin.

[3] Either the S-1 or S-3, Republic-Seitz Filter Corp., Newark, N. J., is suitable. (Mention of this company does not constitute an endorsement of its products by the Department of Agriculture to the detriment of other companies not mentioned.)

TABLE IV.—OPTICAL ROTATION OF RUTIN (MOISTURE CONTENT 7.7%) IN VARIOUS SOLVENTS AND SOLVENT-WATER MIXTURES

Solvent	Solvent Strength, % by Wt.	Optical Rotation $[\alpha]_D^{30° \pm 1°}$ F. at Rutin Concentration of 0.5000 Gm./100 cc.	1.000 Gm./100 cc.	2.000 Gm./100 cc.
Ethanol	99.7	+11.46	+12.19	+13.26
Ethanol	95.0	+ 6.77	+ 7.58	...[a]
Ethanol	49.9	− 9.10	...[a]	...[a]
Methanol	99.8	+ 4.63	+ 5.35	+ 6.68
Methanol	49.9	− 1.52	...[a]	...[a]
Isopropanol	98.2	+ 3.52	...[a]	...[a]
Isopropanol	49.1	− 4.60	...[a]	...[a]
n-Butanol	99.9	+20.77	+18.46	+18.25
Acetone[b]	49.6	−16.03	−11.02	...[a]
1,4-Dioxane	100.0	+21.37	...[a]	...[a]
1,4-Dioxane	50.0	− 9.21	−14.73 (−11.98)[c]	−12.52[c]
Pyridine[d]	100.0	−38.35	−38.83	−40.01
Pyridine	80.0	−41.98	−47.10	−48.12
Pyridine	25.0	−33.47	−34.56	−34.06
Acetic acid	99.8	+42.98	+42.25	+37.97
Acetic acid	49.9	− 3.63	...[a]	...[a]

[a] Indicates rutin not soluble to this extent.
[b] Acetone, 99.1%, would not dissolve this rutin in sufficient quantity to make a 0.5% solution.
[c] Values with technical grade 1,4-dioxane.
[d] Since pyridine-rutin solutions react with metals, contact with metal parts of the polarimeter tube must be avoided.

1,4-dioxane was purified according to the method of Fieser (12). The percentage water by weight in each was determined by use of the Karl Fischer reagent.

Procedure.—When possible, a 2% solution of rutin was prepared by dissolving 1.0000 Gm. of rutin in 40 to 45 cc. of solvent (warming when necessary) and, after cooling, diluting to 50 cc. in a volumetric flask. One per cent and 0.5% solutions were prepared by dilution (pipetting 25 cc. and diluting to 50 cc. in a volumetric flask). Where rutin was not sufficiently soluble in a solvent to make a 2% solution, the correct quantity of rutin was weighed directly to prepare the 1% or 0.5% solution. All measurements were made with a sodium light, using a 4-dm. tube in a Bellingham-Stanley polarimeter. The results obtained with a variety of solvents and water solutions of the solvents are presented in Table IV.

Discussion.—The few published values for the optical rotation of rutin are at variance. This disagreement is no doubt attributable to the impure condition of the rutin samples used. Sosa and Plouvier (13) made a study of the optical rotation of *Forsythia viridissima L.* rutin in various solvents but purified the rutin by only three recrystallizations from aqueous solvents. Our experience with *Forsythia* rutin (14) would indicate that their rutin was not free of quercetin and other contaminants (unidentified sterol-like material). In general, their results with "anhydrous rutin" in the solvents pyridine, ethanol, methanol, and dioxane are slightly lower than ours.

From our data the following conclusions with respect to optical rotation may be suggested: of the solvents tried, rutin gives the largest angle of rotation in pyridine (80 or 100%) and glacial acetic acid, and these solvents appear to be the best for measuring optical rotation. With these, 0.5000 to 2.000 Gm./100 cc. concentration of rutin is satisfactory, especially when a 4-decimeter tube is used. The third choice solvent is *n*-butanol in the same concentration range. Rutin was found to be dextrorotatory in neutral (and anhydrous acid) solvent and levorotatory in dilute neutral, acid, and basic[a] solvents.

SUMMARY

The approximate solubility of rutin in various common solvents such as water, methanol, ethanol, isopropanol, and acetone is reported. Curves for the solubility of rutin in water-solvent mixtures are given for solutions at room temperature and near the boiling point. One maximum solubility occurred in the anhydrous solvent, and another at solvent concentration of 50–80%. The minimum solubility occurred at a solvent concentration of 90–98%. Some data are given on the solubility of rutin in glycerol, *n*-propanol, *n*-butanol, 1,4-dioxane, pyridine, and glacial acetic acid.

Data are presented to illustrate the formation of solvates of rutin with methanol, ethanol, *n*-propanol, and acetone. Use has been made of this phenomenon in the development of a method for the purification of rutin. The method is specially effective in the removal of quercetin and minor flavonoid constituents, and is useful in the preparation of rutin suitable for paranteral injection.

The optical rotation of rutin in methanol, ethanol, isopropanol, *n*-butanol, acetone, 1,4-dioxane, pyridine, and glacial acetic acid has been determined. Readings were made with rutin dissolved in pure solvents and water solutions. Rutin was dextrorotatory in neutral and acidic solvents. Dilution with water de-

[a] Earlier in our work on rutin, levorotatory values were obtained for morpholine; these are not reported because the rutin used was not of high purity.

creased the rotation; in 50% dilution, rotation is levorotatory. Rutin was levorotatory in the alkaline solvents used.

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Stability of Vitamin B_{12} Activity in Liver Injection*

By E. M. STAPERT, E. B. FERRER, and L. STUBBERFIELD†

An investigation was made to determine the stability of vitamin B_{12} activity in liver injection preparations. The effects of exposure to air, oxygen, and light were investigated. Stability studies were made at temperatures of 5, 24, 40, and 47°. Deterioration demonstrated by microbiological assays was confirmed by chemical assay.

THE INTRODUCTION of a specific method for the determination of vitamin B_{12} activity (1) made possible a study of the stability of this activity in liver injection. Stability studies being conducted in these laboratories using a microbiological assay procedure with *L. leichmannii* 4797 as the test organism and using aseptic technique (2) indicated that the vitamin B_{12} activity in liver injection preparations was stable for at least ten months at temperatures up to 47°. Occasional discrepancies were found in assays on the same lot of liver injection when variations were introduced in sample handling or in storage containers. Other investigators (3, 4) have also reported considerable variation in the vitamin B_{12} activity of identically labeled liver preparations; therefore, it seemed desirable to investigate the stability of these products with respect to temperature, exposure to air and oxygen, and exposure to light.

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† The authors wish to thank Joan Bedford, Marthe Ann Miller, and Patricia Rohloff for their assistance in conducting the microbiological assays.

EXPERIMENTAL

Materials and Methods.—Samples of U. S. P. liver injection containing 0.5% phenol were used throughout this investigation. Vitamin B_{12} potencies in the first two studies were measured by the *L. leichmannii* 4797 aseptic microbiological method (2). All other assays were made by the U. S. P. assay procedure (1) modified by determining growth turbidimetrically after twenty hours' incubation. Although information has been reported (2, 5) on correlation of the values obtained by the two assay techniques, this was not considered necessary in reporting stability data.

Effect of Temperature.—Samples of Lot A, a liver injection containing 15.5 μg. of vitamin B_{12}/cc., were stored in the dark at 5, 24, and 40°. The containers were 10-cc. rubber-stoppered vials sealed with metal caps. Previously unopened samples were assayed at one, two, four, and ten months. Samples of Lot B, a liver injection containing 12.3 μg. of vitamin B_{12}/cc., were stored in the same type of containers at 24, 40, and 47°. Previously unopened samples were assayed at one, two, three, four, and ten months. The one exception is the assay representing storage at 24° for ten months. This was done on material from the vial which had been opened and partially used for the four-month assay. These data are shown in Fig. 1, examination of which shows that liver injection in these 10-cc. vials retains 85.2% of the vitamin B_{12} potency when stored for ten months at 5 or 24° and 76.8% of the potency when stored at 40 or 47°.

In contrast, the sample which was stored unopened for four months at 24° without deterioration and then held for six additional months as a partially filled vial showed only 42.5% of its vitamin B_{12} activity.

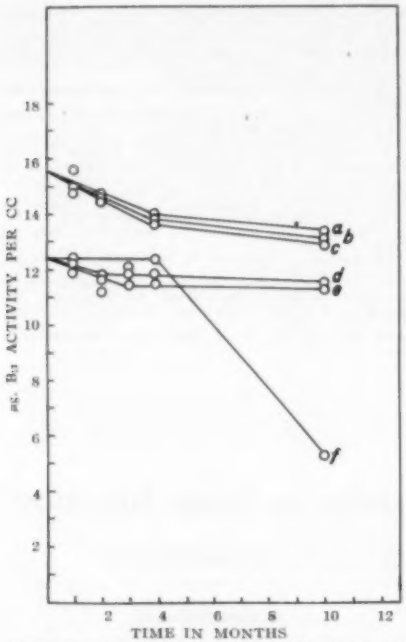

Fig. 1.—Deterioration studies of vitamin B_{12} activity in liver injection. (*a*) Lot A, 5°; (*b*) Lot A, 24°; (*c*) Lot A, 40°; (*d*) Lot B, 40°; (*e*) Lot B, 47°; (*f*) Lot B, 24°.

Effects of Air-Liquid Ratios in Sealed Containers.—Lot C, a 10-U. S. P.-unit liver injection which was filled in 1-cc. ampuls and in 30-cc. vials, assayed 7.4 and 10.9 μg. of vitamin B_{12}/cc., respectively, after storage at 24° for thirty days. Lot D, a 10-U. S. P.-unit liver injection, filled in 1-cc. ampuls and in 10-cc. vials, assayed 3.6 and 7.4 μg. of vitamin B_{12}/cc., respectively, after storage at 24° for three months and 2.2 and 7.2 μg./cc., respectively, after four months' storage at 24°. These values show evidence of greater deterioration in the 1-cc. ampuls than in the 10-cc. or 30-cc. vials. It was evident that 1-cc. ampuls had a greater amount of dead air space, i. e., a greater air-to-liquid ratio than the 10-cc. and 30-cc. vials. Measurements were made to determine the ratio of air-to-liquid in these three container sizes. Average values are shown in Table I.

Factors involved in the deterioration are complex; nevertheless, if exposure to air is a factor, the ratio of the surface area of the interface to liquid volume would be one factor determining the amount of deterioration. The ratio of air volume to liquid volume would also be a determining factor. No attempt was made to establish deterioration rates from these values; but it is apparent that in both instances, the ratio for the 1-cc. ampul is the greatest.

TABLE I.—AIR-TO-LIQUID RATIOS OF INJECTABLE LIVER CONTAINERS

Type Container	Ratio of Air-to-Liquid Volume	Ratio of Air-Liquid Interface to Liquid Volume
1-cc. ampul	0.338	0.519
10-cc. vial	0.172	0.277
30-cc. vial	0.222	0.250

Effect of Exposure to Air.—Lot E, a liver injection containing 20.7 μg. of vitamin B_{12}/cc., was used in a study to determine the rate of deterioration when the liver was exposed to air. Triplicate 50-cc. samples of the liver injection in 125-cc. Erlenmeyer flasks with loose-fitting caps were stored in the dark at 24°. Assays were made daily for seven days, then at twenty-five days and ninety days. After ninety days, the assay value was 2.5 μg. of vitamin B_{12}/cc. These data are shown in Fig. 2. It should be noted that each point on the graph is the average value for assays of 3 samples. As controls for this study, samples of the same lot of liver were stored in the dark at 24° in full, stoppered 125-cc. Erlenmeyer flasks. After five days, the vitamin B_{12} potency of the control was 19.4 μg./cc.; after twenty-five days, 19.4 μg./cc.; and after sixty days, 19.4 μg./cc. These data are also shown in Fig. 2.

Effect of Exposure to Oxygen.—Samples of Lot E (20.7 μg. of vitamin B_{12}/cc.) were agitated in an atmosphere of oxygen for five hours and fifteen hours. The vitamin B_{12} potencies after treatment were 18.3 and 16.3 μg./cc., respectively. A comparison of curves in Fig. 2 shows that increasing the oxygen concentration above that of air does not appreciably alter the deterioration of vitamin B_{12}. The sample that was agitated fifteen hours assayed 13.2 μg. of vitamin B_{12}/cc. after standing four days at 24°. The sample agitated for five hours assayed 11.4 μg. of vitamin B_{12}/cc. after standing fifteen days at 24°. These results approximate the value expected from an air-exposed sample.

Effects of Autoclaving.—When samples of Lot E (20.7 μg. of vitamin B_{12}/cc.) were autoclaved for ten minutes at 15 p. s. i., there was no loss of vitamin B_{12} activity. Upon exposure to air at 24° for twenty-four hours, these autoclaved samples showed no loss in potency. After exposure to air for eight days and fifteen days, the previously autoclaved samples assayed 14.2 and 11.4 μg. of vitamin B_{12}/cc., respectively. Referring to Fig. 2, it can be seen that after a lag period of three to four days, the deterioration rate of the autoclaved samples approximated that of the untreated samples. Considering oxygen to be the active ingredient in air, the lag in deterioration of the vitamin B_{12} activity in the autoclaved samples could be caused by depletion of dissolved oxygen, either physically or by other reactions that compete with the one causing destruction of the vitamin B_{12} analogs.

Effect of Light.—A sample of Lot E liver injection (20.7 μg. of vitamin B_{12}/cc.) was exposed to intense light for two hours. The sample in a full, stoppered container was placed in a transparent water bath and kept at 24°. The light was supplied by two 500-watt incandescent lamps placed 8 in. from the sample. The potency after two hours' illumination was 17.6 μg. of vitamin B_{12}/cc. (85%). A second sample was held at 24° in diffused daylight.

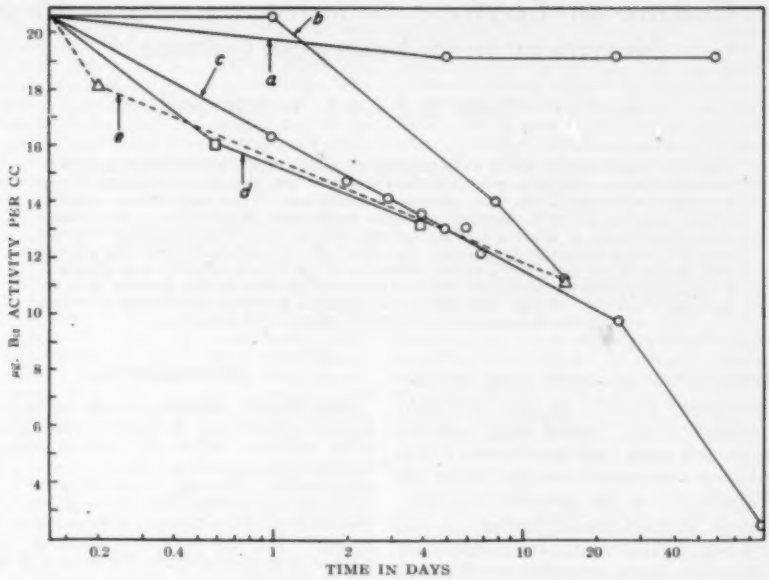

Fig. 2.—Deterioration of vitamin B_{12} activity in liver injection. (*a*) Controls; (*b*) Autoclaved, then air-exposed; (*c*) Air-exposed; (*d*) 15 Hrs. oxygen, then air-exposed; (*e*) 4 Hrs. oxygen, then air-exposed.

After seven days, this sample assayed 19.3 μg. of vitamin B_{12}/cc. or the same as the sample stored in the dark.

Comparison of Chemical and Microbiological Assays.—A liver injection, Lot F, containing 10.4 μg. of vitamin B_{12}/cc. by microbiological assay and 9.2 μg./cc. by chemical assay (4) was stored in a half-filled container for forty-two days at 24° and then was reassayed. The microbiological assay was 6.6 μg./cc, and the chemical assay was 5.5 μg./cc. The percentage deterioration was 36.5% by microbiological assay and 40.0% by chemical assay.

DISCUSSION

Data have been presented to show that the vitamin B_{12} activity of liver injection stored in full, sealed containers is stable over a range of temperatures from 5 to 47°; exposure to air or oxygen causes a loss of 87.5 per cent of the vitamin B_{12} potency in ninety days. These data are shown graphically in Figs. 1 and 2. Other experimental results show that the amount of air present in sealed containers may alter the vitamin B_{12} stability of these products.

Experiments in which samples of liver injection were exposed to oxygen showed that increasing the oxygen concentration over that of air did not increase the rate of deterioration. This is interpreted as indicating that after a certain concentration of oxygen is present, the deterioration rate is dependent on the vitamin B_{12} analog concentration. This is substantiated by the experiments with the air-to-liquid ratio in various containers, and by the experimental results obtained with autoclaved samples.

The effect of light on the vitamin B_{12} activity of liver injection has been studied. Exposure to daylight does not cause appreciable deterioration in seven days.

The loss of vitamin B_{12} activity from exposure to air shown by microbiological assays was confirmed by chemical assay.

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The Culture of *Claviceps purpurea*. I. Growth and Nutrition in Submerged Culture*,†

By V. E. TYLER, Jr.,‡ and A. E. SCHWARTING

Claviceps purpurea was found to be capable of prolific mycelial development in submerged culture. Of the numerous carbon and nitrogen sources investigated, it was found that mannitol (2 per cent) and casein hydrolysate (1 per cent) incorporated in a basic nutrient solution allowed maximum production of mycelium. An average yield of 1.48 Gm. of dry weight of growth per 100 ml. of medium in a period of seven days following minimum inoculation was obtained. A correlation between growth and change in the hydrogen ion concentration of the culture medium was observed. It appears possible to predict the optimal fermentation time in any medium by a determination of *p*H. Strain variation of the organism has been emphasized as an important factor in the submerged culture of *C. purpurea*.

BREFELD (1) is credited with being the first investigator to study the growth of *Claviceps purpurea* (Fries) Tulasne under artificial conditions, and other early contributors to the knowledge of this subject included Meyer (2) and Engelke (3). As the medicinal importance of the ergot alkaloids steadily increased during the first half of this century, the possibility of their production under controlled conditions of biosynthesis prompted numerous workers (4–13) to investigate certain nutritional and environmental factors in relation to their effect on growth and alkaloid production of the organism. The scope of such investigations was limited by the slow growth which the organism exhibited on surface cultures, and published results in relation to alkaloid production are not in agreement.

With the advent of submerged culture growth techniques for microorganisms, and the rapid production of material made possible by its application, it appeared desirable to undertake a re-examination of this problem to obtain more specific information.

In preparation for a detailed study of the mechanism of alkaloid biosynthesis in *C. purpurea*, it was necessary to develop a nutrient medium in which the organism could produce a near-maximum amount of growth in a relatively short period of time. Specific information regarding the application of submerged culture technique to the growth of *C. purpurea* is lacking in the literature. A previous investigation (13) has shown, however, that the concentration of inorganic constituents in the culture medium is not critical.

* Received April 25, 1952, from the College of Pharmacy, University of Connecticut, Storrs.

† Abstracted from a thesis submitted to the Graduate School of the University of Connecticut by V. E. Tyler, Jr., in partial fulfillment of the requirements for the degree of Master of Science.

‡ Eli Lilly Research Fellow 1950–1951. The work reported in this paper was supported by grants from Eli Lilly and Company, Indianapolis, Ind.

EXPERIMENTAL

Basic Nutrient Solution.—In this study a basic nutrient solution was designed to which could be added different carbon and nitrogen sources in varying concentrations in order to arrive at a favorable medium. Although the micronutrient requirements for *C. purpurea* have not been conclusively demonstrated, trace elements were routinely incorporated. The micronutrient constituents of the solution were patterned after the formula of Beadle and Tatum (14).

The basic nutrient solution, including micronutrients, was composed as follows:

NH_4NO_3	1.0	Gm.
KH_2PO_4	2.0	Gm.
$MgSO_4 \cdot 7H_2O$	0.5	Gm.
$CaCl_2$	0.1	Gm.
$FeSO_4 \cdot 7H_2O$	0.1	Gm.
$Na_2B_4O_7 \cdot 10H_2O$	0.088	mg.
$CuSO_4 \cdot 5H_2O$	0.392	mg.
$MnCl_2 \cdot 4H_2O$	0.072	mg.
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.037	mg.
$ZnSO_4 \cdot 7H_2O$	8.81	mg.
Distilled Water, a sufficient quantity, To make	1000	ml.

This solution, with the exception of the ferrous sulfate, was prepared as a stock solution in tenfold concentration and diluted with distilled water prior to use, at which time the ferrous sulfate, carbon, and additional nitrogen sources were added.

D-Glucose, sucrose, maltose, lactose, D-levulose, D(+)-galactose, and mannitol, as well as mixtures of glucose and maltose and glucose, maltose, and levulose, were selected as carbon sources. These had all been shown by previous investigators (4–11, 13) to be utilized readily by the organism in various types of surface culture. Casein hydrolysate (enzymatic), soy hydrolysate (enzymatic), peptone corn steep liquor solids, yeast extract, malt extract, asparagin, potassium nitrate, and a mixture of alanine, glycine, and serine were employed as nitrogen sources.

The single carbohydrates were routinely employed in concentrations of 1% and 2% with the promising nitrogen sources. If the carbon source was particularly well utilized, as evidenced by a high yield of mycelium, its concentration was increased in further trials. Those nitrogen sources which failed to give

substantial yields when paired with a good carbon source were subsequently discarded. As in the case of the carbohydrates, if a nitrogen source proved particularly effective, its concentration was raised, and further experiments were conducted in combination with an increased carbon concentration. Since all of the media contained ammonium nitrate in the basic nutrient solution, its presence is not included in the final nitrogen tabulations. Similarly, the amount of available carbon present in some of the organic nitrogen sources has not been taken into account in the carbohydrate concentrations.

The strain of *C. purpurea* used in the course of these experiments was originally obtained from a sclerotium of domestic rye ergot of the 1948 crop, supplied by the Universal Grain Products Company of Dassel, Minn. A pure culture was obtained by cutting back the outer pigmented layers under aseptic conditions and subsequently removing and transferring a small portion of the inner pseudoparenchyma to a slant tube containing nutrient agar medium. In a few weeks the surface of the slant was covered with numerous white hyphal strands.

At the conclusion of certain preliminary studies of approximately six months duration, the strain was tested for the retention of its parasitic properties. An aqueous suspension of the growth was prepared and applied to the tops of the open flowers of rye plants which had reached the proper stage of development in a greenhouse. At the end of two weeks, infection was in evidence. One flower had reached the honeydew stage, and several others were seen to be in earlier stages of development.

Conidia isolated from this honeydew developed slowly on a nutrient agar medium, and transfers taken from this culture were used as inoculum in all experiments reported in this paper. It was found that a more uniform quantity of inoculum could be obtained if the organism to be used for this purpose was maintained in submerged culture. This was accomplished by preparing flasks containing the basic nutrient solution in combination with 2% mannitol and 1% soy hydrolysate. These flasks were inoculated from cultures on solid nutrient agar and shaken for approximately 168 hours. At this time, typical feathery mycelial growth pervaded the media, and the flasks were stored in a refrigerator. Stock cultures so prepared remained viable for at least six months and allowed nearly uniform inoculation with a transfer loop.

One hundred-milliliter portions of the nutrient solution containing the specified quantities of carbon and nitrogen sources were placed in 500-ml. Erlenmeyer flasks. The *p*H of each medium was determined with a Beckman H-2 *p*H meter; the flasks were then stoppered with nonabsorbent cotton, sterilized in an autoclave at 15 pounds pressure for fifteen minutes, and allowed to cool. After inoculation, the flasks were placed in racks on a reciprocating shaking machine which performed about 150 excursions per minute. Operating at this speed, the liquid media tended to swirl in a circular motion in the flask, and splashing was reduced to a minimum.

Periodic observations indicated that maximum growth was reached in a majority of the media after a fermentation period of six to eight days at room temperature, after which time growth weight either remained nearly constant or started to decrease slowly as autolysis began. Consequently, a period of approximately seven days was established as most convenient, and the flasks were removed from the shaker after about 168 hours.

It was subsequently determined that an equivalent weight of growth could be obtained in a much shorter period of time (approximately seventy-two hours) if a quantity of inoculum, greater than that retained by a transfer loop, was utilized. For this purpose a special transfer cup, with a capacity approximating 0.8 ml., was prepared and is now in current use in this laboratory. However, all of the results reported in this paper were obtained from cultures inoculated by means of a transfer loop.

Typical growth consisted of feathery mycelial strands varying in color from white to shades of brown and gray, depending upon the nitrogen source present in the medium. Occasionally the growth assumed distinct ball shapes, usually accompanied by long thick strands of mycelium.

After fermentation, the volume of medium and growth in each flask was again measured to determine if loss by evaporation or splashing was excessive, and the *p*H of the medium was then determined. Growth was collected on a tared sintered-glass crucible, washed with distilled water to remove undissolved solid material from the medium, and dried in an oven at 40° for at least twenty-four hours. The crucible and growth was then transferred to a vacuum desiccator and dried over phosphorus pentoxide for an additional forty-eight hours. Dry growth weight was then determined by weighings made on an analytical balance.

Results of the systematic studies of carbon and nitrogen nutrition are summarized in Table I. The recorded figures are in most instances the average of two separate trials, but in some cases as many as five trials have been utilized in computing the dry weight of growth. Two of the carbon sources, glucose and mannitol, were tested at increased concentrations in combination with good nitrogen sources. These results are presented in Table II. Three of the better nitrogen sources were tested in varying concentrations, in combination with levulose and mannitol, to determine if nitrogen might be a limiting factor in certain media; results are listed in Table III.

Carbohydrate Nutrition.—Glucose proved to be a fair carbon source, allowing average to poor growth with the sources of nitrogen enumerated. It is evident from Table II that in all of the glucose media, available nitrogen was the limiting factor, since an increase in carbohydrate concentration did not result in increased weight of growth in the same fermentation time. Growth remained nearly constant until the highest concentration (16%) of glucose was reached, at which time it fell off sharply, probably due to an unfavorable osmotic pressure. The hydrogen ion concentration of all glucose media increased during fermentation, regardless of the nitrogen source.

Sucrose proved to be an erratic carbon source. At concentrations of 1%, it supported some growth in every instance; but when increased to 2%, no significant growth took place with two of the five nitrogen sources tested. As with glucose, the acidity of the medium increased during the fermentation period.

TABLE I.—DRY WEIGHT OF GROWTH (GM./100 ML.) of C. PURPUREA GROWN IN SUBMERGED CULTURE IN THE BASIC NUTRIENT SOLUTION INCORPORATING VARIOUS CARBON AND NITROGEN SOURCES

	Soy Hydrolysate, 0.5%	Casein Hydrolysate, 0.5%	Yeast Extract, 0.5%	Peptone, 0.5%	Corn Steep Solids, 0.5%
Glucose, 1%	0.61	0.47	0.41	0.52	...
Glucose, 2%	0.41	0.44	0.33	0.25	0.11
Sucrose, 1%	0.52	0.13	0.44	0.70	0.22
Sucrose, 2%	0.00	0.20	0.93	0.35	0.00
Maltose, 1%	0.25	0.18	0.15	0.05	0.11
Maltose, 2%	0.25	0.25	0.19	0.05	0.15
Lactose, 1%	0.17	0.09	0.12	0.02	0.06
Lactose, 2%	0.20	0.13	0.13	0.04	0.12
Levulose, 1%	0.65	0.67	0.48	0.60	0.60
Levulose, 2%	1.04	0.95	0.91	0.74	0.80
Galactose, 1%	0.16	0.13	0.09	0.05	0.08
Galactose, 2%	0.20	0.16	0.11	0.09	0.04
Mannitol, 1%	0.58	0.60	0.54	0.48	0.67
Mannitol, 2%	1.19	1.22	0.89	0.27	0.00
Glucose, 1% and Maltose, 1%	0.37	0.08	0.33	0.54	0.56
Glucose, 1%, Maltose, 0.5%, Levulose, 0.5%	0.60	0.70	0.62	0.55	0.25

Maltose was an inferior sugar, allowing only scant submerged growth with all of the nitrogen sources. Again, as in the case of glucose, nitrogen availability was clearly limiting, but with maltose the culture medium became more basic during the culture period.

Lactose was also very poorly utilized by *C. purpurea* in each of the combinations tested. Although growth increased slightly when the concentration was raised from 1 to 2%, this advance was not appreciable. As with maltose, the *p*H of the medium increased in every instance during fermentation.

The most versatile sugar proved to be levulose. Although surpassed by mannitol in some instances, it gave fair to good growth when paired with any of the nitrogen sources. Increasing the concentration from 1 to 2% gave a considerable increase in growth weight with the better nitrogen sources, and this increase was appreciable in every case. Apparently nitrogen availability was not limiting at a concentration of 2% levulose, for 0.5% concentrations of both soy hydrolysate and yeast extract gave better yields of mycelium than 1% concentrations of either of these nitrogen sources. Change in *p*H was not consistent in the levulose media, but seemed to be dependent upon the concentration of the sugar and the identity of the nitrogen source.

The most poorly utilized of all the carbon sources tested was galactose. Growth was very poor with each of the five nitrogen combinations, and an increase in the sugar concentration from 1 to 2% did not result in an appreciable increase in mycelium. With the exception of the 2% concentration with 0.5% corn steep solids, media incorporating this sugar tended to become more alkaline during fermentation. This lone exception may be accounted for by the extremely sparse growth in that medium.

Mannitol gave superior growth, yielding the highest dry weight of mycelium of any of the carbon sources in certain instances. It was not, however, as consistent as levulose with all nitrogen sources. In the case of those nitrogen sources which afforded good utilization of the mannitol, growth was approximately doubled when the concentration of mannitol was increased from 1 to 2% (Table I). A slight additional increase was noted when the nitrogen source was also raised from 0.5 to 1% (Table III). Mannitol concentrations of 4%, 8%, and 16% gave somewhat erratic results (Table II); but even at these high concentrations, mannitol seemed to be well tolerated by the organism. This is in contrast to results obtained with glucose media. As with levulose, it appeared difficult to correlate the observed *p*H changes with the carbon source. Increase or decrease in hydrogen ion con-

TABLE II.—EFFECT OF INCREASED CARBOHYDRATE CONCENTRATIONS ON THE DRY WEIGHT OF GROWTH (GM./100 ML.) OF C. PURPUREA

Concn. Glucose, %	Casein Hydrolysate, 0.5%	Concn. Mannitol, %	Soy Hydrolysate, 0.5%
1	0.47	1	0.58
2	0.44	2	1.19
4	0.39	4	0.67
8	0.40	8	1.20
16	0.15	16	0.95

TABLE III.—VARIATION OF DRY WEIGHT OF GROWTH (GM./100 ML.) OF C. PURPUREA IN RESPONSE TO CHANGES IN CONCENTRATION OF THE NITROGEN SOURCE

	%	Levulose, 2%	Mannitol, 2%
Soy hydrolysate	0.25	0.68	0.84
	0.5	1.04	1.19
	1.0	0.99	1.32
Yeast extract	0.25	0.69	...
	0.5	0.91	...
	1.0	0.63	...
Casein hydrolysate	0.25	...	1.02
	0.5	...	1.22
	1.0	...	1.48

centration seemed to depend upon the concentration of mannitol as well as the identity and concentration of the nitrogen source.

A combination of glucose and maltose, 1% of each, proved to be an average carbon source in several nitrogen combinations. With one exception (0.5% corn steep solids) these media became more acid during fermentation. Subsequently, a mixture of glucose (1%), maltose (0.5%), and levulose (0.5%) was incorporated in media containing different nitrogen sources. This combination proved to be more readily utilizable by the organism, but was still inferior to either mannitol or levulose when used singly in corresponding amounts. With a single exception (0.5% soy hydrolysate) the acidity of the media increased during the fermentation period. It is interesting to note that the addition of this small amount of levulose to the glucose-maltose combination resulted in a complete reversal of efficiency of the five nitrogen sources listed.

In summary, mannitol was the best carbon source tested, followed by levulose which proved to be more versatile in regard to nitrogen combinations. Sucrose and the glucose-maltose-levulose combination supported rather good growth, with glucose and the glucose-maltose mixture almost as good. Maltose, lactose, and galactose were inferior carbon sources.

The Effect of Growth on the Hydrogen Ion Concentration of the Culture Medium.—In view of the erratic changes in hydrogen ion concentration observed in the different media at the end of the culture period, an experiment was carried out to determine the effect of growth upon the *p*H of the culture medium during fermentation. For this trial, the basic medium incorporating 0.5% casein hydrolysate and 1% glucose was selected. A number of 500-ml. Erlenmeyer flasks, each containing 100 ml. of the medium, were prepared in the manner previously described. At regular intervals during the fermentation, flasks were removed from the shaking machine, the *p*H of the medium was determined, and the growth was filtered out, dried, and weighed in the usual manner. After growth had passed the maximum weight (221 hours), 5 ml. of a sterile aqueous solution containing 1 Gm. of glucose was added to each of half the remaining flasks. The sampling process was then continued with both the original flasks and those containing the added sugar, until a total fermentation time of 525 hours was reached. The results of this experiment are represented in Fig. 1.

Assuming the results obtained with glucose to be typical of the various other carbon sources used in these studies, a probable explanation for the observed *p*H changes in all media may be formulated from this data. The *p*H of the medium increased slightly up to the point where growth became evident and then fell rapidly as the growth weight increased rapidly. Whenever a poorly utilized carbon source was employed, no substantial quantity of mycelium was formed, and the small increase in growth weight took place very slowly. Under these conditions it may be assumed that the *p*H curve did not fall off sharply, as with glucose, but either continued upward in a fairly straight line or dipped slightly and then once more began to ascend.

As rapid mycelial development ceased, (75–150 hours in the case of glucose), the *p*H of the medium, having reached its lowest point, began to curve upward until about 200–220 hours, when it exceeded the original *p*H of the medium. As fermentation continued, the *p*H value became constant at a neutral or slightly alkaline level.

Those media containing well-utilized carbon sources demonstrated an increase in acidity when harvested at about 168 hours. Although the point of maximum growth had been reached at this time, the *p*H curve was slowly ascending after maximum decline but was still well within the acid range.

Variations in the *p*H of those media containing superior carbon sources are probably explained by the fact that in such media the *p*H rose more rapidly following its maximum decrease, and after a fermentation period of 168 hours was either slightly above or below the original hydrogen ion concentration. With glucose this occurred after a somewhat longer period of time.

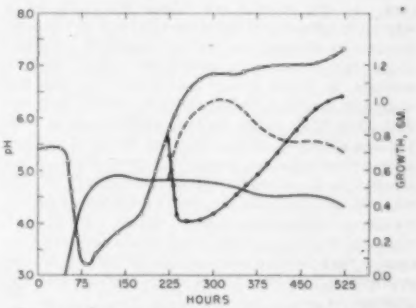

Fig. 1.—The influence of carbon availability on the growth and *p*H of submerged cultures of *C. purpurea*.

———— Growth weight (original glucose concn.)
– – – – – – Growth weight (glucose added)
—○—○—○— *p*H (original glucose)
—●—●—●— *p*H (glucose added)

When an additional gram of glucose was added to the medium after maximum growth had been reached with the original concentration, the growth weight increased, giving a curve similar to the one obtained initially. Simultaneously the *p*H decreased and then rose, giving a curve similar to the initial *p*H curve.

From this evidence it may be postulated that the organism growing in submerged culture converted the carbohydrate into some organic acid which was then metabolized. As this was utilized, the hydrogen ion concentration of the medium decreased until a *p*H of approximately 7 was reached. Then, as the organism began to undergo autolysis, basic substances were released from the mycelium resulting in a still further rise in the *p*H of the medium.

The practical value of observation of *p*H change is that it renders possible the estimation of approximate time required for the organism to attain maximum growth in any specific medium.

Nitrogen Nutrition.—In combination with the better carbon sources, casein hydrolysate and soy hydrolysate gave the best results, followed by yeast extract, corn steep solids and peptone (Table I).

Additional nitrogen sources, including potassium nitrate (0.1%), asparagin (0.25%), alanine-glycine-serine (0.25% each in combination), and malt extract (0.5%), proved to be extremely poor nitrogen sources in combination with 1% and 2% concentrations of glucose, and were discarded as routine possibilities.

Increases in growth weight were noted when the concentration of casein hydrolysate or soy hydrolysate was raised from 0.25 to 0.5% and to 1%. Similar results were not observed with yeast extract. Growth weight increased when the concentration was raised from 0.25 to 0.5%, but at 1% the weight of growth was less (Table III).

DISCUSSION

At the time of completion of this work, no study of the nutritional requirements of *C. purpurea* in submerged culture had been reported in the literature. In the interim one paper has appeared in which the influence of aeration-agitation of *Claviceps* cultures by shaking has been investigated. Using a single culture medium containing 1.25% each of maltose and yeast extract and 0.125% of peptone, originally intended for stationary culture, Sim and Youngken (15) report average yields ranging from 119.3 to 204.3 mg. of mycelium per 60 ml. of medium after a fermentation period of seven days. Since identity and concentration of ingredients of this medium vary from the one used in this investigation, no direct comparison can be drawn. In general, however, such low yields tend to confirm the conclusion that maltose is an inferior carbon source and peptone and yeast extract only average nitrogen sources.

The rapid and abundant growth attained by *C. purpurea* in submerged culture renders it impossible to make direct comparisons of the results obtained in this study with those reported by previous workers for surface cultures. In most of the early experiments on solid media, growth was of necessity estimated by visual inspection only, because of the difficulty involved in quantitative separation of the mycelium and substrate. However, the experimental results reported here are in rather close agreement with those of most previous investigators, with some notable exceptions.

Michener and Snell (13) demonstrated that mannitol and maltose were utilized very slowly in surface cultures, but both eventually allowed rather good growth. In submerged culture mannitol was utilized rapidly and efficiently, but maltose proved to be a rather poor carbon source. Kirchhoff (5) indicated that galactose allowed growth comparable to dextrose, levulose, and mannitol. In this study, galactose was decidedly inferior.

Several authors including Kirchhoff (5), De Tempe (10), and Michener and Snell (13) have indicated that asparagin was a good nitrogen source for growth of *C. purpurea*, but in this submerged culture study it was very poorly utilized. McCrea (6) incorporated malt extract as well as peptone in her medium, but in this study, malt extract alone gave disappointing results.

With these exceptions, the nutritional requirements for this organism in submerged culture seem to be in rather good agreement with those reported by other investigators for surface growth.

These requirements doubtless vary considerably with the composition of the inorganic nutrient medium as well as the efficiency of aeration. A third (and necessarily the most important) variation noted in submerged saprophytic cultures of *C. purpurea* is strain difference. It has been noted repeatedly in the course of work with this organism over the past three years that different isolates and different subcultures of the same isolate exhibit great differences in rate of growth, appearance, pigment formation, and other aspects of metabolism. At present this factor remains an unsolved problem, but work is continuing in this laboratory toward a solution of this and other aspects of the metabolism of *C. purpurea* in submerged culture, with particular emphasis on biosynthetic pathways, end products, and analysis. A paper presenting new techniques for chromatographic separation of the ergot alkaloids has recently been published (16).

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Observations on the Acute Toxicity of Two Chlorinated Phenols*

By J. MEHSEN JOSEPH

Acute toxicity studies on methyl chlorothymol and *p*-chloro-*m*-xylenol indicated low toxicity, which is rarely observed with chlorinated phenolic compounds. The MLD and the LD_{50} of methyl chlorothymol for mice were both found to be 0.78 gram. The toxicity of *p*-chloro-*m*-xylenol was found to be almost four times less than that of methyl chlorothymol.

METHYL CHLOROTHYMOL (MCT) is a potent germicidal and fungicidal agent which has shown great promise as a preservative for cosmetics, pharmaceuticals, and biologicals. Methyl chlorothymol is chemically 2-tertiarybutyl-4-chloro-5-methylphenol and has a molecular weight of 198.5. It is insoluble in water, inorganic acids, and glycerin, but soluble in alcohols, benzene, ethers, pine oil, castor oil, Spans, Tweens, and dilute NaOH and KOH. Pure MCT is nonirritating and nonsensitizing. The phenol coefficient (FDA method) is tremendously influenced by the nature of the diluent, ranging from 130 to 250; pine oil and terpineol exalt the coefficient, while excessive alkaline carriers tend to depress it.

p-Chloro-*m*-xylenol (PCMX) is a very safe and versatile germicide which is easily compatible with pharmaceuticals and cosmetological products. It is chemically 3,5-dimethyl-4-chlorophenol, with a molecular weight of 156. PCMX is a white crystalline solid which is readily soluble in 95 per cent alcohol at room temperature but insoluble in water. It is nontoxic, noncorrosive, nonirritating, and nonsensitizing (1). The phenol coefficient as determined by the Rideal-Walker method varies from 40 to 80. According to Zondek (2), PCMX is very useful in the treatment of urogenic infections (pyelitis, cystitis), being bactericidal toward staphylococci and *Proteus vulgaris*, and bacteriostatic toward *Escherichia coli*. Lum (3) found that 1.0 per cent PCMX was able to keep a vaccine sterile for a period of twelve hours when exposed to dusty air. For nearly twenty years, PCMX has been used in Germany and England as a general antiseptic, germicide, and fungicide. In this country within recent years, PCMX has been used in fungicides, vaginal jelly, deodorant bath salts, and a trade-marked general antiseptic.

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Because of the increasing tendency to exploit the use of these two effective germicides (MCT and PCMX) in the manufacture of soaps, shaving creams, cosmetics, pharmaceuticals, etc., it is worth while to present data concerning the acute toxicity of these compounds.

EXPERIMENTAL

Acute Toxicity of Methyl Chlorothymol for Mice.—Albino mice, weighing 25 Gm. each, were divided into 6 groups of 6 mice per group. Each animal in group 1 was injected intraperitoneally with 0.5 cc. of pure MCT, each in group 2 with 0.6 cc., each in group 3 with 0.7 cc., each in group 4 with 0.8 cc., each in group 5 with 0.9 cc., and each in group 6 with 1.0 cc. The mice were observed constantly for a period of five days and the time of death was recorded. From the data obtained, the minimum lethal dose (MLD) and the lethal dose for 50% of the experimental animals (LD_{50}) were calculated according to the Reed-Muench formula (4).

Acute Toxicity of *p*-Chloro-*m*-Xylenol for Mice.—Since PCMX is practically insoluble in water, several aqueous suspensions of fine crystals carrying 2.0 Gm., 2.5 Gm., 3.0 Gm., 3.5 Gm., and 4.0 Gm., respectively, were used for injection. Albino mice, weighing 25 Gm. each, were divided into 5 groups of 6 mice per group. Each animal in group 1 was injected intraperitoneally with 2.0 Gm. of PCMX, each in group 2 with 2.5 Gm., each in group 3 with 3.0 Gm., each in group 4 with 3.5 Gm., and each in group 5 with 4.0 Gm. These animals were examined daily for a period of five days. The MLD and LD_{50} were calculated (4).

RESULTS AND DISCUSSION

The results presented in Table I indicate that MCT is of very low toxicity for mice. Both the MLD and the LD_{50} were found to be 0.71 cc. or 0.78 Gm. per 25 Gm. mouse. In other words, the test animal could tolerate up to 30.8 Gm. per Kg. body weight. The quantity of this germicide required for the preservation of various biologicals and pharmaceuticals is infinitely small in comparison to its toxic dose, and therefore it could be considered safe for external and internal use.

It is apparent that PCMX is less toxic than MCT. The MLD of PCMX was found to be 3.0 Gm. per 25 Gm. mouse, or 120 Gm. per Kg. The LD_{50}, according to the Reed-Muench formula (4), was found to be 2.88 Gm. (Table II). A compound of such low toxicity and high germicidal power, plus its other merits, is probably the nearest approach to the ideal preservative. Previous tests indicate that PCMX is a valuable germicide for use in surgical soaps, in cold sterilization of operating room equipment, and for the preservation of vaccines and various biologicals.

TABLE I.—ACUTE TOXICITY OF METHYL CHLOROTHYMOL FOR MICE

Dosage Cc.	Ratio of No. Died to No. Injected	Accumulative Totals			
		Died	Survived	Deaths	% Mortality
1.0	6/6	20	0	20/20	100
0.9	6/6	14	0	14/14	100
0.8	5/6	8	1	8/9	89
0.7	3/6	3	4	3/7	43
0.6	0/6	0	10	0/10	0
0.5	0/6	0	16	0/16	0

TABLE II.—ACUTE TOXICITY OF *p*-CHLORO-*m*-XYLENOL FOR MICE

Dosage, Gm.	Ratio of No. Died to No. Injected	Accumulative Totals			
		Died	Survived	Deaths	% Mortality
4.0	6/6	16	0	16/16	100
3.5	6/6	10	0	10/10	100
3.0	4/6	4	2	4/6	67
2.5	0/6	0	8	0/8	0
2.0	0/6	0	14	0/14	0

CONCLUSIONS

These two chlorinated phenols, methyl chlorothymol and *p*-chloro-*m*-xylenol, possess exceedingly low toxicities. Both the MLD and the LD_5 for MCT were found to be 0.78 Gm. per 25 Gm. mouse, while the MLD and the LD_{10} for PCMX were 3.0 Gm. per 25 Gm. mouse and 2.88 Gm., respectively. In view of the acute toxicity studies and germicidal power, these compounds should have extensive application.

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Micro Synthesis with Tracer Elements. IX. The Synthesis of Ethylenediamine Tetra-(2-C^{14}-Acetic) Acid*,†

By ARTHUR MURRAY, III, and ANTHONY R. RONZIO

THE SODIUM SALT of ethylenediamine tetraacetic acid (Versene) possesses the property of strongly reducing ion activity through the formation of stable soluble complexes with many cations. A sample of this compound, labeled with C^{14}, was desired for metabolic and equilibrium studies.

Only two practical syntheses of the acid are generally known. Exploratory small scale experiments employing the reaction between ethylenediamine and sodium chloroacetate (1) proved to be unsatisfactory because of low yield and considerable by-product formation. The carboxymethylation of ethylenediamine described by Smith, *et al.* (2), although very time-consuming, proved to be satisfactory in a 3-millimole synthesis, provided the recommended amount of sodium cyanide was doubled and added incrementally to offset its loss through hydrolysis.

The principal competing reactions for formaldehyde (3) are those involving the use of ammonia leading to formation of hexamethylenetetramine, glycine, and di- and tri-glycolamino acids. Clearly, anything facilitating removal of

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† This investigation was carried out under the auspices of the Atomic Energy Commission.

ammonia from the reaction as soon as it is formed is a requisite if high yields are to be attained. This became increasingly evident as smaller scale runs were made in which the volume of solvent could not be conveniently reduced below that imposed by the C^{14}-formaldehyde preparation.

Despite claims (2) that the rapid, high-yield industrial procedure (4) is not adaptable to small-scale preparations, it was felt that rapid formaldehyde addition, continuous ammonia removal, and elevated temperature could be successfully utilized. When ammonia was expelled by running the reaction under reflux (atmospheric pressure, b. p. 107° instead of 60°) and with a stream of nitrogen scrubbing out the mixture, a series of 21 experiments indicated that the yield was not appreciably affected by either rate of formaldehyde addition or alkali concentration, but was definitely dependent upon scale of run (at constant total aqueous volume). Investigation proved that ethylenediamine, upon which yield was based, was not being lost through entrainment in the gas stream. Ammonia removal was further facilitated by active distillation and by keeping the volume of reaction solvent small during formaldehyde addition. To fix the ethylenediamine in a non-volatile form, it was carboxymethylated prior to distillation with slightly more than one equivalent of formaldehyde in the cold (no ammonia evolution) and then was hydrolyzed under conditions of reflux. With this modification, the average yield (six-millimole scale) rose from 21 to 76 per cent.

EXPERIMENTAL

The carboxymethylation was run in a 100-ml., round-bottomed, two-necked flask equipped with a reflux condenser, a magnetic stirrer, and a self-venting addition tube of 15-ml. capacity which fed the reagent through a fine drip-tip into a nitrogen stream emerging beneath the surface of the reaction mixture. The rate of nitrogen flow was adjusted so that 10 bubbles per twenty seconds passed through a water-filled U-tube.

To a stirred, ice-cooled mixture of 0.3596 Gm. of ethylenediamine (0.00597 mole, freshly distilled from sodium), 7 ml. of water, 0.30 Gm. of sodium hydroxide, and 0.0179 moles of sodium cyanide, a 19.5-ml. aliquot of aqueous C^{14} formaldehyde solution (0.00748 mole) was added at a uniform rate over a one-hour period. This formaldehyde solution (5) was made by diluting the unanalyzed catalytic oxidation product (assumed yield 79%) from 1.473 millimoles of C^{14} methanol (8.00 mc.) with 0.0170 moles of nonisotopic formaldehyde to a volume of 54.5 ml. (0.0209 mole total). No ammonia was evolved up to this point. The ice bath was removed and stirring was continued overnight at room temperature. Hydrolysis was then completed by heating under reflux (ten hours). The reflux condenser was then removed, and the mixture was concentrated to approximately 10–15 ml. To the boiling reaction mixture was added 1 ml. of a solution of 0.0269 mole of sodium cyanide and 0.30 Gm. of sodium hydroxide made up to 7.0 ml. with water. The remainder of the C^{14} formaldehyde solution was added at the same slow rate as before, while distillation of the reaction mixture was continued at such a rate as to maintain a small volume. One milliliter of the cyanide solution was added after the addition of every 5 ml. of formaldehyde solution. The reaction was completed by continuing in the same manner with: (*a*) 0.0239 moles of sodium cyanide and 0.15 Gm. of sodium hydroxide diluted to 8 ml. with water (2 ml. per addition), and (*b*) 0.01194 mole of nonisotopic formaldehyde in 20 ml. of solution. Complete hydrolysis was assured by boiling the reaction mixture under reflux until no further ammonia evolution occurred. The combined distillates were found to be contaminated with very little radioactivity.

Considerable silicic acid was precipitated from the colorless reaction mixture by neutralizing with concentrated hydrochloric acid, boiling for a few minutes, and cooling. The filtered silicic acid was thoroughly washed with water and the washings were added to the filtrate which was then concentrated to a volume of 10–15 ml. An additional small amount of flocculent silicic acid was removed at room temperature by filtering and washing with five 1-ml. portions of water. The product was precipitated from solution (25–30 ml.) by adjusting to *p*H 1.20 with concentrated hydrochloric acid and allowing the solution to stand at 0° for about twenty-four hours. The product was collected by filtering the solution through a chilled, sintered-glass Buchner funnel and washing the filtrate with small portions of cold water until it gave no test for chloride ion. The colorless powder weighed 1.2695 Gm. (72.8%) after drying for three hours at 100°. The millimolar radioactivity was determined to be 1.44 mc. by combustion and by counting of barium carbonate plates with a Geiger-Muller tube having a thin mica end-window.

Three trial runs by this procedure gave 73.2 to 80.7% yields. No residue was left upon ignition. Ethylenediamine tetraacetic acid decomposed without melting at 243° and could not be sublimed (.01 mm., 140–200°). A recovery of 95% was obtained from 0.6 Gm. of pure compound dissolved in alkaline silicate solution. The best recrystallization medium for ethylenediamine tetraacetic acid was water, from which it separated as microscopic needles. The water solubility is 0.53 mg./ml. at 30° and 6.7 mg./ml. at 92°.

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North Dakota Volatile Oils. III. Comparative Studies on Coriander Oil*

By CLIFTON E. MILLER†

A report on the physical and chemical properties of two samples of coriander oil is presented. It was observed that the oil obtained from the dried ripe fruit contained about 20 per cent more linalool than the oil from the entire plant. Both oils were found to contain a considerable quantity of aldehydes, calculated as decyl aldehyde.

THE volatile oil program under way at North Dakota Agricultural College has already been reported (1, 2). This paper presents, as part of that program, a comparative study of cordiander oils obtained from the dried ripe fruit of *Coriandrum sativum* and also from the partially field-dried whole plant, including the fruit.

The coriander oils used in this study were obtained from the dried ripe fruit and from the whole plants of *Coriandrum sativum* which were grown during the summer of 1950 at the College Experimental Farm. The coriander fruit was harvested in late September approximately one hundred days after planting. The plants were threshed in the field with a calculated yield of 980 pounds of dried ripe fruit per acre. Steam distillation of the fruit yielded 0.38 per cent volatile oil, hereafter called Sample 1, on a moisture-free basis. No cohobation of the aqueous distillate was carried out. As a matter of academic interest, a portion of the partially field-dried whole plant, including the fruit, was steam distilled in the usual manner and this oil is referred to as Sample 2. No attempt was made to calculate the yield of this sample of oil.

EXPERIMENTAL

Physical Properties.—The oil from the fruit (Sample 1) was a pale yellow liquid with a sweet odor and a characteristic taste. Sample 2 was green in color and the odor was strong and woody. The physical constants of the oils (after drying over anhydrous sodium sulfate) are recorded in Table I; for comparison, average values of coriander oil U. S. P. are included.

Chemical Properties.—The acid value and ester value were determined on the two oil samples following the official procedures as outlined in U. S. P. XIV (3). The linalool and aldehyde contents were determined according to the Essential Oil Association method (4). Average results of the several determinations are given in Table II.

TABLE I.—PHYSICAL CONSTANTS OF CORIANDER OILS

	Sample 1	Sample 2	U. S. P. Oil
d^{25}	0.8688	0.8505	0.863–0.875
n_D^{20}	1.4613	1.4520	1.4620–1.4720
$[\alpha]_D^{25}$	+10.1°	+6.2°	+8.0°–15.0°
Solubility in 70% alcohol	3 volumes	3 volumes	3 volumes

TABLE II.—CHEMICAL CONSTANTS OF CORIANDER OILS

	Sample 1	Sample 2
Acid value	Negligible	Negligible
Linalool, %	80.50	61.42
Linalyl acetate, %	3.58	19.46
Aldehyde, % (as decyl aldehyde)	11.62	22.49

Distillation Analysis.—Distillation analysis of the two oils as a means of identification of the terpenes present gave disappointing results. This may possibly be explained by the low hydrocarbon content (5). Only one terpene, α-phellandrene, was isolated as its maleic adduct. The presence of an unreported terpene, with a maleic adduct melting point of 138–140°, was observed in both samples. The lack of information concerning maleic adducts of terpenes has already been noted (1).

CONCLUSIONS

1. A comparative study of coriander oils from the dried ripe fruit and from the entire plant including the fruit of *Coriandrum sativum* has been made, and certain differences have been noted.

2. The physical and chemical properties of the oil from the entire plant are similar to those of coriander herb oil reported by Guenther.

3. An oil yield of 0.38 per cent was obtained from the dried ripe fruit.

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Water Sorption Studies on Rutin and the Determination of Moisture*

By J. NAGHSKI,[1] E. F. MELLON, A. H. KORN, and C. L. OGG†

Vapor phase water sorption studies were made on rutin of pharmaceutical grade and on highly purified rutin. The absorption and desorption curves for moisture at various relative humidities demonstrate the extremely hygroscopic nature of rutin. Several procedures for the determination of moisture in such a hygroscopic material were investigated. High temperature, vacuum, and an extremely dry atmosphere in the oven were found to be essential to insure complete drying. Special precautions were necessary to prevent rehydration of the samples after drying. A recommended procedure is described for determining moisture in rutin.

THE DRYING of rutin, either for obtaining anhydrous samples for analytical studies or for obtaining a value for moisture, has always been a difficult problem because of the extremely hygroscopic nature of the compound. Perkin (4, 5) found that rutin dried over concentrated sulfuric acid three weeks lost 5.76 per cent water ($2H_2O$ = 5.44%); after additional drying at 130°, it lost 1.49 per cent ($^1/_2$ H_2O = 1.43%); and finally after drying at 160° it lost an additional 1.67 per cent ($^1/_2$ H_2O = 1.47%), making a total of 3 molecules of water. He also observed that upon exposure to air the dried rutin quickly absorbed water. The extreme hygroscopic nature of rutin was demonstrated again in this laboratory during carbon and hydrogen analyses. Small dry samples of rutin picked up more than 2 per cent of their weight of water in the few seconds in which they were exposed to room air of 50 per cent relative humidity while being transferred from a micro weighing pig to the combustion tube. To obtain anhydrous samples for analysis, Porter, *et al.* (6), found it necessary to dry rutin under vacuum at 130° in the presence of magnesium perchlorate and in a specially designed drying vessel.

EXPERIMENTAL

Water Sorption Studies.—To determine the normal degree of hydration of rutin, vapor phase absorption studies were made using the technique reported for water absorption studies on proteins (2).

Rutin of N. F. IX (3) quality was further purified by crystallizing five times, alternately from absolute ethanol and water. The moles of water absorbed per mole of flavonol at various degrees of relative humidity are presented in Fig. 1.

The highly purified rutin absorbed up to 3.1 moles of water when exposed to high humidities while the N. F. rutin absorbed up to 4 moles of water. The absorption curves for the two types of rutin are different. The curve for highly purified rutin shows a large uptake of water at a low humidity while that for the N. F. grade rutin shows a gradual uptake with increasing humidity. The desorption curves for the two rutins are similar, both showing little loss of moisture until extremely low humidities are reached. These results show clearly why it is difficult to obtain an anhydrous sample.

To determine the effect of possible impurities on the absorption of water by rutin, the water absorption of quercetin[1] was investigated. The results are also presented in Fig. 1. Quercetin absorbed two moles of water, which is in agreement with

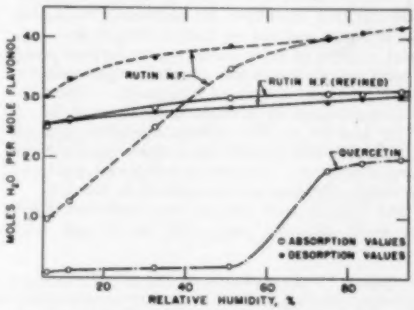

Fig. 1.—Water sorption isotherms for rutin and quercetin showing degree of hydration in relation to relative humidity.

reports in the literature (1, 8). The shape of the curve suggests that the presence of quercetin may be the cause for the lag in water absorption by the N. F. rutin. However, since less than 5% of quercetin is present in the N. F. rutin (3) it cannot account for the results observed. If the data for water absorption by the rutin and quercetin are plotted on a weight basis (Fig. 2) it will be observed that the N. F. rutin absorbs more water

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[1] Quercetin was recrystallized three times from 70% ethanol.

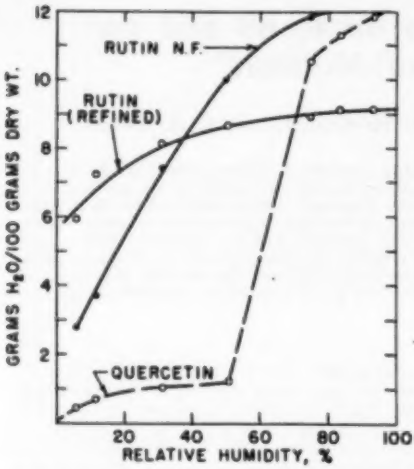


Fig. 2.—Water absorption of rutin and quercetin.

than even the quercetin. Either it contains small amounts of extremely hygroscopic impurities, or the presence of the quercetin disrupts the crystal structure sufficiently to permit the accumulation of free water. The latter is the more likely explanation.

Determination of Moisture in Rutin.—The results of sorption studies presented above were used to develop a simpler and more efficient procedure for the determination of moisture in rutin. To determine the best conditions for accurately measuring the moisture content of such a hygroscopic material, a series of experiments was performed, using a rutin sample which was shown by spectrophotometric analysis (6) to be of high purity. The rutin was equilibrated in a room maintained at 75 ± 1° F. and 50 ± 2% relative humidity, and all experiments were carried out in rooms having these same conditions. The drying conditions used and the results obtained are summarized in Table I.

The vacuum oven used in these experiments was the small chamber type with outlet and inlet valves on opposite sides of the oven and a vacuum gauge mounted in the self-sealing door. The inlet was connected to a drying train consisting of a gas wash bottle with fritted glass gas disperser, half filled with concentrated sulfuric acid, followed by a 2-ft. length of 60 mm. O. D. glass tubing containing a 14-in. section of anhydrous calcium sulfate followed by a 6-in. section of phosphorus pentoxide on pumice stone chips. With the drying train and separate outlet and inlet valves, the vacuum chamber can be swept continuously with dry air and the vacuum can be released with dry air at the end of the drying period. To sweep the oven during drying, an air inlet valve was opened slightly so that about 25 ml. of air per minute was drawn through the drying train. The principles involved in this procedure for drying were discussed by Willits (9).

The special weighing bottles (Fig. 3) used in experiments 3, 8, 9, and 11 were made by drilling a 2-mm. hole through the 24/12 ground glass joints of the Parr type weighing bottles. Drilling was done with the cap in place so that the holes in the cap and bottom coincided. When drying a sample in the special bottle the hole was left open to allow the moisture to escape from the sample chamber by diffusion through the opening. Upon removing the bottles from the oven the caps were immediately turned to close the hole. Thus the amount of moist laboratory air coming in contact with the sample after drying was limited to that which would diffuse through the hole during the short time required to remove the bottle from the oven and turn the cap.

The desiccant used in experiments 4 to 9 was anhydrous magnesium perchlorate placed in a shallow tray in the bottom of the vacuum oven We do not recommend magnesium perchlorate for general use as an oven desiccant where there is a possibility of its picking up organic vapors. In these experiments, however, where it was known that the only volatile material present was water, magnesium perchlorate was considered to be a safe substitute for the anhydrous barium oxide used in the National Formulary procedure (3).

The data (Table I) show that drying in the air oven at 110° (experiment 1) left approximately 2% moisture in the rutin. The usual vacuum oven drying technique (experiment 2) at 110° gave a

TABLE I.—EVALUATION OF DRYING CONDITIONS FOR THE DETERMINATION OF MOISTURE IN RUTIN

Experiment No.	110° C.—16 Hrs. 1	2	3	125° C.—16 Hrs. 4[a]	5	6	7	8	9[b]	10	11[c]
Air oven	x	...	...	...	...	...	...	...	...	...	...
Vacuum oven	...	x	x	x	x	x	x	x	x	x	x
Desiccant in oven	...	...	...	x	x	x	x	x	x	...	...
Oven swept with dry air	...	...	x	...	...	...	...	...	...	x	x
Vacuum released with dry air	...	...	x	...	...	x	x	x	x	x	x
Vacuum released with room air	...	x	...	x	x	...	...	...	...	...	...
Parr type weighing bottle	x	x	...	x	x	x	x	...	...	x	...
Special weighing bottle	...	...	x	...	...	...	...	x	x	...	x
Sample weight (Gm.)	0.1	0.1	0.1	0.5	0.1	0.5	0.1	0.5	0.1	0.1	0.1
Av. % loss on drying	6.44	7.59	8.17	8.21	8.08	8.29	8.32	8.37	8.39	8.31	8.42
Standard deviation	0.11	0.06	0.15	0.01	0.21	0.02	0.04	0.02	0.04	0.03	0.01

[a] National Formulary procedure.
[b] Drying conditions same as recommended in reference 6.
[c] Recommended procedure (0.5 Gm. samples should be taken when a macro balance is used).

moisture figure about 0.8% low. Only when some more effective measure was taken to remove the water vapor from the vacuum oven chamber was the true moisture value approached.

In experiment 3 the moisture was removed from the vacuum chamber by a dry air sweep and the dry sample was protected by releasing the vacuum with dry air and by using the special weighing bottles. The moisture value of 8.17 is much better than that of experiment 2, but comparison with the value obtained by the same technique at 125° (experiment 11) shows that even under the best conditions a temperature of 110° is not quite sufficient. Experiment 4 is the same as the National Formulary procedure except that magnesium perchlorate was substituted for barium oxide. This substitution should have negligible effect on the results since the two desiccants are comparable, the barium oxide being recommended because of the possible hazard associated with the perchlorate. This procedure gave a value of 8.21% which was higher than the 8.08% obtained in experiment 5, where only 0.1-Gm. samples were used. This difference is due probably to the samples picking up moisture between the end of the drying phase and the final weighing. The amount of moisture absorbed would depend more on the degree of exposure of the sample to room air than on the sample weight. To reduce this possible source of error, the air used to release the vacuum in experiments 6 and 7 was passed through a drying train. The results of 0.1- and 0.5-Gm. samples were now in good agreement and were higher than those for the two preceding experiments. The procedure of using the dry air release was adopted. To protect the samples from moist air when the oven was opened, the further refinement of using the special weighing bottles was tested in experiments 8 and 9. Comparing the results obtained with those of experiments 6 and 7 shows that this expedient increased the moisture value obtained by approximately 0.1%.

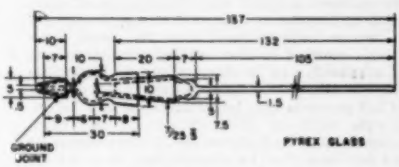
It has been shown that it is necessary to release the vacuum with dry air; consequently, a drying train will need to be connected to the oven. Experiments 10 and 11 were conducted to compare the effectiveness of using dry air from this train to sweep the water vapor from the oven with that of placing a desiccant in the vacuum chamber. The values for experiments 10 and 7, which differ only in the method used for removing water vapor from the vacuum chamber, were 8.31 and 8.32, respectively. Similarly, the values for experiments 11 and 9 were 8.42 and 8.39, showing that the desiccant and dry air sweep methods were equally effective. The method of sweeping with dry air is preferred because it avoids the danger and trouble of placing a desiccant in the oven.

Increases in loss on drying resulting from using the dry air vacuum release and the special weighing bottle were both of the order of 0.1%. To determine if these differences were statistically significant, Student's t values (7) calculated from the data were compared with the critical t values. The significance of the effect of the special weighing bottle on the results was determined by pooling the data from experiments 8, 9, and 11 and comparing the mean with that of the pooled data from experiments 6, 7, and 10. This pooling of the data was done to obtain larger and more reliable samples for the statistical comparison and was justified because the sample size and the method for desiccating the oven chamber had no significant effect on the values under the conditions of these six experiments. The calculated t value, 2.54, was greater than the critical $t_{0.05}$, 2.10, showing that the difference was significant at the 95% level and the increase in the moisture values for rutin resulting from the use of the special weighing bottles was real. When the t test was used to compare the results of experiment 4 with the pooled data from experiments 6 and 7, releasing the vacuum with dry air was found to give significantly higher results even at the 99% level than those obtained when room air was used to release the vacuum [t (calcd.) = 5.44; $t_{0.01}$ = 3.06].

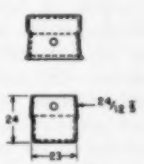
The standard deviation values shown in Table I were generally lower for 0.5-Gm. samples and this would have been much more pronounced had not all weighings been made to the nearest 0.01 mg., using a semimicro balance. The relatively standard deviations for experiments 1, 2, 3, and 5 are also a good indication that drying was not complete.

It has been assumed throughout this discussion that there was sufficient rutin to permit using at least 0.1-Gm. samples. If less material is available or if a small dry sample is desired for analysis, the special charging tube, Fig. 3, described by Porter, *et al.* (6), should be used. The dry air sweep (experiments 10 and 11) was equally as satisfactory as the desiccant method (experiments 6 to 9) used by Porter for drying rutin in the charging tubes.

To determine if the prolonged drying at 125° had any deleterious effect on the rutin, two samples dried according to the conditions of experiment 11 were analyzed by the spectrophotometric method of Porter, *et al.* (6), and found to be 99.6% and 99.9% pure. Two other samples of the same rutin were analyzed for purity without being dried and



CHARGING TUBE



WEIGHING BOTTLE

ALL DIMENSIONS IN MILLIMETERS

Fig. 3.—Special weighing bottle and charging tube used for determining moisture in hygroscopic materials.

the results were calculated to the moisture-free basis using the moisture value from Experiment 11. The values obtained, 99.8% and 100.1%, were within the normal experimental error of the results obtained on the dried samples and indicate that rutin is not measurably degraded by heating at 125° for sixteen hours. Rutin samples dried at 125° for sixteen, thirty-two, and forty-eight hours all showed the same per cent loss in weight, which is further evidence of the heat stability of rutin.

The following procedure is recommended for the determination of moisture in rutin. Dry the special weighing bottle (Fig. 3) in a vacuum oven for one-half hour and cool to room temperature in a desiccator containing an efficient desiccant (anhydrous calcium sulfate or better). Place approximately 0.5 Gm. of rutin in the tared bottle and weigh to the nearest 0.1 mg. Turn the bottle cap so that the hole is open and place in a vacuum oven at 125°. With a continuously operating vacuum pump, draw moisture-free air from the drying train described above through the oven at approximately 25 ml./min., controlling the air flow with the inlet valve. Continue sweeping with dry air for sixteen hours (overnight), close the outlet valve, and bring the oven to atmospheric pressure by bleeding dry air into the chamber (about ten min.). Remove the sample from the oven, immediately turn the cap of the weighing bottle to close the hole and place in the desiccator until it cools to room temperature. Weigh to the nearest 0.1 mg. and calculate the percentage loss in weight of the sample.

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Infrared Analysis of Active Ingredients in Ointments

I. Atropine Sulfate and Scopolamine Hydrobromide*

By WILLIAM H. WASHBURN

An infrared method for the analysis of alkaloids in ointments is described which appears to be an improvement over the usual titrimetric method in that smaller concentrations of alkaloids can be determined and specifically identified. Atropine sulfate in the amount of 0.5 per cent may be determined in a 2-Gm. sample with an accuracy of ±2 per cent. Scopolamine hydrobromide in the amount of 0.2 per cent may be determined in a 3-Gm. sample with an accuracy of ±5 per cent.

THE ANALYSIS of alkaloids in ointments has been the subject of few investigations. Titrimetric methods for the determination of alkaloids are not very practical for the assay of small concentrations; they lack the specificity inherent in infrared analysis. Recently, several studies have been made on the application of infrared spectroscopy to the investigation and determination of alkaloids (1–3). In our laboratories, infrared methods which are specific and sensitive for small amounts of alkaloids in ointments have been developed.

* Received July 5, 1952, from the Control Department, Abbott Laboratories, North Chicago, Ill.

EXPERIMENTAL

In order to investigate the bands available for quantitative determination, pure spectra of both atropine base and scopolamine base were recorded. Atropine, the spectrum of which is shown in Fig. 1, was prepared by evaporating 1 cc. of a 2% carbon tetrachloride solution on a silver chloride plate of 1 mm. thickness. Scopolamine (Fig. 2), which is a viscous liquid, was prepared by pressing a drop of material between two rock salt plates. Per cent transmittance curves were constructed from the single beam record, using the device of Willis (4).

In ointments, water-soluble salts of both atropine and scopolamine are employed. This necessitates conversion of the salts to free bases to make possible quantitative infrared work with a suitable nonpolar solvent. In the case of both atropine and scopolamine, carbon tetrachloride was found to be the solvent of choice. For atropine, the band at 8.56 μ was found to be the one of preference in quantitative work; in the case of scopolamine, the band at 11.21 μ was selected. The ointment base in these preparations consists of lanolin, petrolatum, and mineral oil.

Standard working curves were constructed as follows: several portions of U. S. P. grade atropine sulfate were dissolved in water and the solutions were made slightly alkaline with 15% ammonium

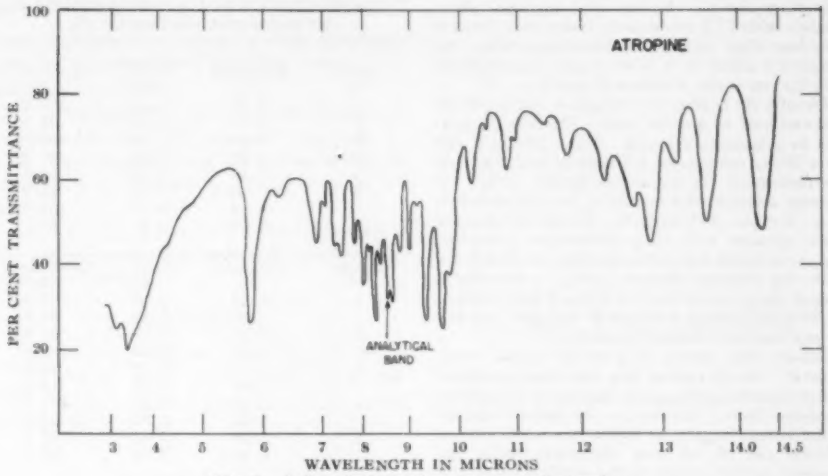

Fig. 1.—Infrared absorption spectrum of atropine.

hydroxide. The atropine was then extracted with four 10-cc. portions of chloroform, and the chloroform was evaporated to dryness. With carbon tetrachloride as solvent, the residues were diluted to known concentrations; a cell of 0.5 mm. thickness was employed to note the absorbancy at 8.56 μ. Absorbancies of solutions in the concentration range of 2–10 mg./cc. were determined and the working curves constructed by plotting absorbancy versus known concentration (Fig. 3).

Exactly the same procedure was used in constructing a working curve for scopolamine hydrobromide (Fig. 4) with the exception that the aqueous phase was made alkaline with 1 *N* sodium hydroxide rather than with 15% ammonium hydroxide, and optical density measurements were made at 11.21 μ.

The measurements in this study were made with a Perkin-Elmer model 12B infrared recording spectrometer equipped with sodium chloride optics and a three-speed slit drive.

Quantitative Infrared Determination of Atropine Sulfate 0.5% in Ointment.—Transfer about 2 Gm. of ointment (accurately weighed) to a suitable flask. Dissolve in 15 cc. of ether and transfer to a separatory funnel. Rinse the flask with two 10-cc. portions of ether and transfer to the separatory funnel. Extract with five 10-cc. portions of water, (rinsing each portion first through the original flask) and collect the aqueous phase in a second

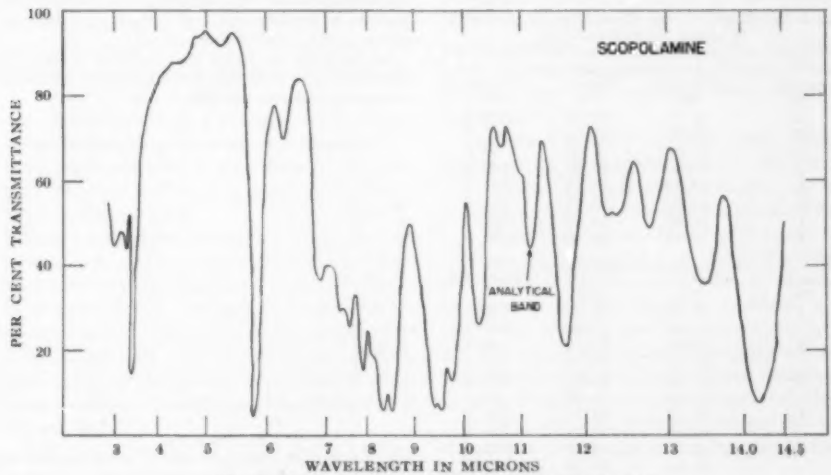

Fig. 2.—Infrared absorption spectrum of scopolamine.

separatory funnel. Render the aqueous phase alkaline with 15% ammonium hydroxide. Extract with four 10-cc. portions of chloroform, collect the chloroform phase in a 50-cc. flask, and evaporate it to dryness under a stream of nitrogen.

Dissolve the residue by adding 5 cc. of 2% sulfuric acid and heat on a steam bath. Transfer the solution to a separatory funnel. Rinse the flask with three 10-cc. portions of 1% sulfuric acid, and add the rinsings to the separatory funnel. Wash the aqueous solution with one 20-cc. portion and three 10-cc. portions of chloroform. Render the aqueous phase alkaline with 15% ammonium hydroxide, and extract with four 10-cc. portions of chloroform. Filter the combined extracts through a chloroform-soaked pledget of cotton into a small flask, evaporate to dryness under a stream of nitrogen, and dry for one hour in a vacuum desiccator.

Dissolve the residue in 2 cc. of carbon tetrachloride. (If the residue does not dissolve entirely in five minutes, apply gentle heating to the tightly-stoppered flask.) Determine the optical density of the sample solution at 8.56 μ in a sealed sodium chloride cell of 0.5 mm. thickness. Refer the observed optical density to the working curve and determine the concentration of the solution. Confirm the identity of atropine by running a qualitative curve on this solution from 8 to 9 μ.

In Table I are listed the results of a series of determinations on ointments of known strength and on commercial preparations.

Quantitative Infrared Determination of Scopolamine Hydrobromide 0.2% in Ointment.—Transfer about 3 grams of ointment (accurately weighed) to a suitable flask. Dissolve in 20 cc. of ether and transfer to a separatory funnel. Rinse the flask with two additional 10-cc. portions of ether and add the rinsings to the separatory funnel. Extract with five 10-cc. portions of water (rinsing each portion first through the original flask) and collect the aqueous phase in a second separatory funnel. Render the aqueous phase alkaline with 1 *N* sodium hydroxide, and extract with four 10-cc. portions of chloroform. Wash the chloroform phase with 10 cc. of 0.05 *N* sodium hydroxide and drain the chloroform solution into a suitable flask. Evaporate to dryness under a stream of nitrogen and dry under vacuum for one hour. (Additional purification of scopolamine is not necessary as it is in the determination of atropine, because the absorption of the ointment base is negligible at 11.21 μ whereas it is significant at 8.56 μ.)

Dissolve the residue in 1 cc. of carbon tetrachloride. (If the residue does not dissolve entirely in five minutes, apply gentle heating to the tightly stoppered flask.) Determine the optical density of the sample solution at 11.21 μ in a sealed cell of 0.5 mm. thickness. Refer the observed optical density to the working curve and determine the concentration of the solution. To confirm the identity of scopolamine, run a qualitative infrared curve on this solution from 11–12 μ.

In Table II are listed the results of a series of determinations on ointments of known strength and on commercial preparations.

TABLE I.—RESULTS OF ANALYSES OF ATROPINE SULFATE OINTMENT 0.5%

Sample No.	Laboratory Preparations: Theoretical, Mg.	Found, Mg.	Recovery, %
1	10.5	10.56	100.6
2	10.80	11.00	101.9
3	10.00	9.94	99.4
4	10.00	10.20	102.0
5	10.32	10.40	100.8
6	10.62	10.78	101.5
7	10.30	10.60	102.9
8	10.10	10.30	102.0

Sample No.	Commercial Ointments: Theoretical, Mg.	Found, Mg.	Label Claim, %
1	10.24	10.50	102.5
	10.00	10.48	104.8
2	10.01	10.10	100.9
	10.11	10.70	105.8
3	10.13	10.36	102.2
	10.11	10.06	99.5
4	10.12	10.74	101.2
	10.03	10.28	102.5
5	9.94	10.32	103.8
	10.03	10.30	102.7

TABLE II.—RESULTS OF ANALYSES OF SCOPOLAMINE HYDROBROMIDE OINTMENT 0.2%

Sample No.	Laboratory Preparations: Theoretical, Mg.	Found, Mg.	Recovery, %
1	6.30	6.31	100.2
2	6.70	6.40	95.5
3	6.20	6.48	104.5
4	6.50	6.25	96.2
5	6.46	6.36	98.5
6	6.20	5.90	95.2
7	6.09	6.20	101.8
8	6.05	5.91	97.7

Sample No.	Commercial Ointments: Theoretical, Mg.	Found, Mg.	Label Claim, %
1	6.22	6.06	97.4
	6.20	5.88	94.8
2	6.13	5.87	95.8
	6.02	5.93	98.5
3	6.04	5.42	89.7
	6.03	5.57	92.4
4	6.28	6.45	102.7
	6.40	5.95	93.0
5	5.99	5.43	90.7
	6.02	5.63	93.5

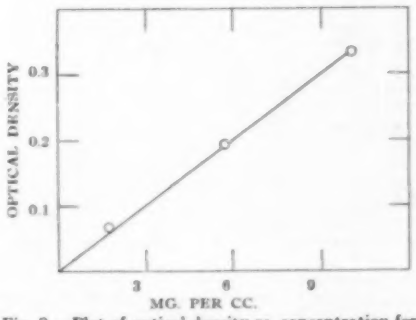

Fig. 3.—Plot of optical density vs. concentration for atropine sulfate at 8.56 μ.

SUMMARY AND CONCLUSIONS

1. An infrared method has been developed for the determination of small amounts of alkaloids in ointments.

2. Reproducibility for the determination of atropine sulfate 0.5 per cent is ±2 per cent.

3. Reproducibility for the determination of scopolamine hydrobromide 0.2 per cent is ±5 per cent.

4. The method is specific in that each alkaloid may be positively identified by its characteristic infrared absorption.

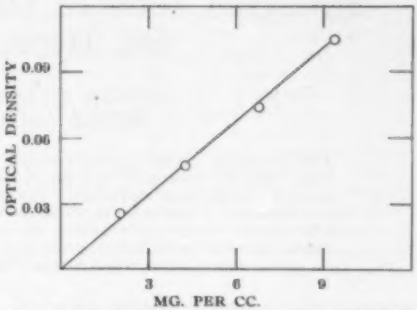

Fig. 4.—Plot of optical density *vs.* concentration for scopolamine hydrobromide at 11.21 μ.

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1953 Iodine Research Award Nominations Requested

Nominations are now being received by the AMERICAN PHARMACEUTICAL ASSOCIATION for the 1953 Chilean Iodine Educational Bureau Award recognizing outstanding research in the chemistry and pharmacy of iodine and its compounds as applied in pharmacy or medicine. Any member of the ASSOCIATION may propose a nominee by submitting eight copies of each of the publications to be considered in the competition, a biographical sketch of the nominee including date of birth, and a list of his publications. Eight copies of the nomination must be submitted to Robert P. Fischelis, Secretary of the AMERICAN PHARMACEUTICAL ASSOCIATION, 2215 Constitution Ave., N. W., Washington 7, D. C. To be eligible for the 1953 Award, nominations must be received on or before February 1, 1953.

A nominee must be a resident of the United States or Canada. He must have accomplished outstanding research in the chemistry or pharmacy of iodine and its compounds as applied in pharmacy or medicine.

During the period covered by the nomination the nominee shall have been actively engaged in, shall have completed, or shall have published a report upon the line of investigation for which the award is made. During the period of two years prior to the date of nomination, the nominee shall not have been engaged in research under the sponsorship of the Chilean Iodine Educational Bureau, Inc.

The award consists of $1,000 and a diploma setting forth the reasons for selection of the recipient. It may be presented annually at the annual meeting of the ASSOCIATION.

The recipient will deliver a paper or lecture upon the subject of his scientific work at the meeting at which the award is conferred. His paper, or address, will then be published in the JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION. In addition to the sum of the award, the recipient will receive an allowance of not more than $250 to defray his expenses in attending the meeting.

The recipient will be selected by an award committee which is appointed by the chairman of the ASSOCIATION's Council. The present committee includes Justin L. Powers, chairman; Louis Gershenfeld, Charles O. Wilson, Harvey B. Haag, Heber W. Younken, John C. Krantz, and Frank O. Taylor.

The award is now in its fifth year. The recipient of the first award was Dr. William T. Salter, Yale University pharmacologist; the second recipient was Dr. George M. Curtis, Chairman of the Department of Surgical Research and Professor of Surgery at Ohio State University, and the third was Dr. C. P. Leblond, Professor of Anatomy, McGill University, Montreal, Canada. The fourth award was shared jointly by Dr. George Moore of the University of Minnesota Medical School and Dr. Moses Ashkenazy, Chief of Neurosurgery, Veterans Hospital, Houston, Tex., and was conferred during the 1952 Convention of the AMERICAN PHARMACEUTICAL ASSOCIATION.

Synergism between Histamine, Antihistamines, and Hypnotic Drugs*,†

By J. L. AMBRUS, C. M. AMBRUS, C. A. LEONARD, C. E. MOSER and J. W. E. HARRISSON‡

The synergism between antihistamines and the barbiturate Evipal was studied in mice. Similar synergism was found between histamine and barbiturates and between antihistamines and ether. The possible clinical usefulness of these findings is discussed. Thephorin, in mice, shows a synergistic effect with barbiturates or ether similar to that of Benadryl; yet according to clinical reports, Thephorin is rather a cerebral stimulant in humans. It is thus believed that this procedure is not suitable to assay sedative side effects of antihistamines experimentally.

MANY antihistamines cause drowsiness, dizziness, and sedation in patients. Dews and Graham (1) demonstrated similar effects in experimental animals; Winter (2) showed that, in guinea pigs and in mice, antihistamines prolong the hypnotic effect of barbiturates. Comparing four different antihistamines, Winter found that the extent of this synergism is proportional to the degree of sedation reported on the individual antihistamines in clinical literature.

The aim of the present study was to investigate whether, on the basis of the findings of Winter (2), it would be possible to work out a convenient assay procedure to test the sedative side effects of antihistamines; to investigate whether antihistamines, besides being synergistic with barbiturates, have a similar effect with general anesthetics, e. g., ether; and finally, to study the effect of histamine itself on the hypnotic effect of barbiturates.

METHODS

Swiss female mice of our own colony were used, maintained on Rockland Complete Rat Diet and water *ad lib.* and kept in air conditioned quarters at 21° C.

Evipal sodium[1] (Hexobarbital) was injected intraperitoneally in a dose of 100 mg./Kg. Each group of about 10 mice was observed by one operator from the time after the injection until the appearance of the following reactions was noted:

(*a*) The animal when picked up by its tail and lowered to the table top does not stretch out its front paws as a protection.

(*b*) The rear portion of the animal's body may be turned to the side without the animal correcting this position.

(*c*) The entire animal may be laid on its side.

(*d*) The animal, when laid on its back, remains in this position.

(*e*) The animal turns spontaneously over into normal position, but continues to sleep.

(*f*) The animal starts to crawl (often after a while, it reverts to stage (*e*) for a period, then begins to crawl).

(*g*) The animal walks without signs of uncertainty; its eyes are fully open, its fur appears to be smooth, and there is no remaining sign of drug action.

An attempt was made to evaluate the experimental data on the basis of each of the above mentioned signs. After many trials, it was concluded that the most objective and most reproducible results were obtained by using stage (*e*) (spontaneous return from the back position) as a criterion and graphing the percentage of animals "awake" in each group at intervals of five minutes, as defined by stage (*e*). The time interval between the injection of the hypnotic and the appearance of stage (*e*) was termed "sleeping time."

Ether was administered in the following manner: ether was placed in a jar immersed in a constant temperature water bath of 35°. Compressed air was bubbled through the ether at a rate of 200 bubbles a minute and the ether vapors were conducted into an anesthetizing jar, which was vented (Fig. 1). This procedure provided a high level concentration of ether which appeared to be less variable in effect.

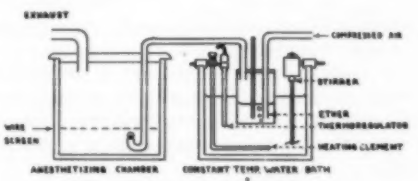

Fig. 1.—Anesthetizing apparatus.

In preliminary experiments it was observed that if an Evipal injection is repeated thirty minutes after complete awaking, no change in "sleeping time" is evidenced, while the effect of a repeated exposure to ether is somewhat more lasting. From day to day, the values observed for individual animals varied considerably. Usually other animals of the same group show variations of similar tendency and magnitude. So far the reason for these

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† Supported (in part) by a grant from Eli Lilly & Co.

‡ The authors wish to acknowledge the assistance of G. V. Rossi.

[1] We are greatly indebted to Winthrop-Stearns, Inc. who supplied us with Evipal.

variations cannot be explained. Because of this, the technique should probably be used only in acute experiments. However, in the cases where drug prolonged the "sleeping time," the same drug in the same group always caused a similar increase in spite of the fact that the absolute values of the group changed considerably from day to day. In all experiments reported here, the control as well as the different experimental groups were tested on the same day.

Histamine and antihistamines were injected subcutaneously fifteen minutes preceding the administration of the hypnotic or anesthetic. Neoantergan (N,N-dimethyl-N'-2-pyridyl-N'-*p*-methoxybenzyl-ethylenediamine maleate); Phenergan (N-dimethylaminopropylphenothiazine hydrochloride); Benadryl (*β*-dimethylaminoethylbenzhydryl ether hydrochloride); Thephorin (2-methyl-9-phenyl-2,3,4,9-tetrahydro-1-pyridindene),[1] and histamine diphosphate were used. The doses always refer to these salts. Solutions were made with physiological saline.

RESULTS AND DISCUSSION

Effect of Different Doses of Histamine on "Sleeping Time."—Data are set forth in Fig. 2. It is to be noted that histamine acts as a synergist with Evipal. As the dose of histamine is increased, the degree of synergism decreases (100 mg./Kg.),

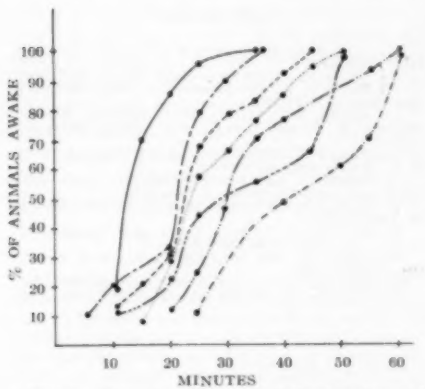

Fig. 2.—Groups received the following doses of histamine subcutaneously: (number of mice used indicated in parentheses) ———— no histamine (27); — — — — 10 mg./Kg. (10); ●·····● 50 mg./Kg. (26); - - - - - - 100 mg./Kg. (24); —·—·—·— 200 mg./Kg. (17); — — — — — — 400 mg./Kg. (9); —·—·—·— 500 mg./Kg. (10). Fifteen minutes after the injection of histamine, all groups received 100 mg./Kg. of Evipal intraperitoneally.

but if the dose is further increased, the synergistic effect again increases. Similar observations were noted in four consecutive experiments. Analogous reactions have been described by Mayer and Brousseau (3) who noted that, in mice, histamine and antihistamines are synergists from the point of view of acute toxicity. Our previous findings from the point of view of body temperature-decreasing effect (4) indicated that histamine and antihistamines were synergists only at certain dose levels; when the dose of antihistamines is increased, antagonism occurs, but a further increase in the dose of antihistamine causes reappearance of synergism. There is no satisfactory explanation for the above observations and it is hoped that the study of such phenomena may ultimately broaden our knowledge of the mechanism of histamine-antihistamine antagonism.

Synergism between Hypnotics and Antihistamines.—In a series of experiments, the effect of antihistaminics on the prolongation of "sleeping time" was compared. With Neoantergan, Benadryl, and Phenergan, similar results were obtained to those of Winter (2); therefore, these are not reported. The effect of different doses of Thephorin are shown in Figs. 3 and 4. Thephorin is recognized as an antihistamine having no or only minimal sedative side effects (5) and a review of the clinical literature supporting this is given by Kallos, *et al.* (6). Indeed, Pennypacker, *et al.* (7) reported that Thephorin exerted rather a cerebral stimulating effect. According to our findings, Thephorin elicits a potent prolongation of "sleeping time" similar to that of Benadryl. It seems, therefore, that the prolongation of the hypnotic effect of barbiturates by antihistamines is not a suitable test for the sedative side effects of the latter drugs.

Synergism between Ether and Antihistamines.—The observed data are graphed in Fig. 4 and appear to be of the same magnitude as the synergism between barbiturates and antihistamines.

Antihistamines may be useful in ether anesthesia for the following purposes: (1) to decrease the dose of ether needed; (2) to decrease mucous secretion and salivation by their atropine-like action (Benadryl

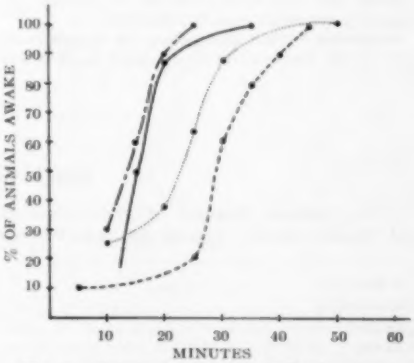

Fig. 3.—Groups received the following doses of Thephorin subcutaneously: (number of mice used indicated in parentheses) ———— no Thephorin (8); — — — — 5 mg./Kg. (17); ●·····● 20 mg./Kg. (8); — — — — 50 mg./Kg. (10). Fifteen minutes after the injection of Thephorin, all groups received 100 mg./Kg. of Evipal intraperitoneally.

[1] We are greatly indebted to the manufacturers for supplying us with the following drugs: Neoantergan and Phenergan (Rhône Poulenc), Benadryl (Parke Davis & Co.) and Thephorin (Hoffman LaRoche).

seems to be especially suitable); (3) to decrease postanesthetic nausea and vomiting (Dramamine was recommended for the purpose by several authors (8, 9); (4) to antagonize possible effect of shock substances (histamine, acetylcholine, etc.) which may be liberated during surgical procedures and which may contribute to the development of postoperative shock; and (5) to decrease cellular and capillary permeability and increase capillary resistance, thus assisting in combating postoperative hemorrhage and shock.

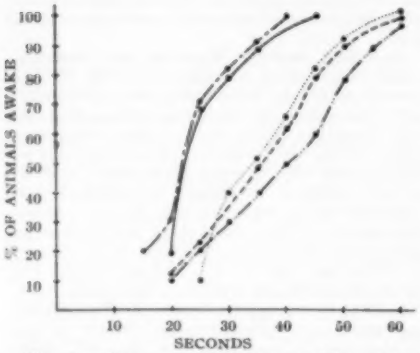

Fig. 4.—Groups received 10 mg./Kg. of the following antihistamines: (number of mice used indicated in parentheses) ·———· no antihistamine (10); ·— — —· Neoantergan (10); ●·····● Thephorin (10); ·— · — · Benadryl (10); ·—·—·—· Phenergan (10). Fifteen minutes after the injection of antihistamine, each group was anesthetized with ether.

It is evident from Fig. 4 that there is synergism between antihistamines and ether, but no relationship exists between the degree of antihistamine activity and the synergism, an observation already evident from the data presented by Winter (2) regarding synergism with barbiturates.

Addendum.—After terminating the experimental part of this study, a lecture delivered by Heinrich (10) came to our attention in which he mentioned that synergism exists between ether and antihistamines. Cronheim and Ehrlich (11) reported recently that synergism was not evidenced between four different N-N substituted N′-N′-dimethylethylenediamines showing antihistaminic potency and the hypnotic effect of pentobarbital.

This observation lends further support to the conclusion that there is no parallelism between the degree of synergistic activity of hypnotics or general anesthetics with antihistamines and the antihistaminic activity of the latter.

SUMMARY

1. Histamine, as well as antihistamines, increases the period of sleep brought about by 100 mg./Kg. Evipal in mice.

2. Antihistamines exhibit synergism with ether. The clinical usefulness of this fact is suggested.

3. The prolonging effect of antihistamines on the hypnotic effect of barbiturates and ether is not parallel with the sedative side effects of the antihistamine preparations as set forth in clinical reports. Neither could there be detected any relationship between antihistaminic activity and synergism with ether or Evipal.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal, and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Thioguaiacol
Thevetigenin
Quercitol
Indican
Brasiline
Cholestane
Coprostane
Epicholesterol
3-Hydroxyisonicotinic acid
2-Mercaptoimidazole
8-Mercaptoquinoline
5-Methyloxine
Oxine-5-carboxylic acid
1,2,3,4-Tetracarboxybutane
2,3,4-Trihydroxybenzalaniline
8-Methoxy quinoline
3,5-Dinitropyrocatechol
2-Chlorofluorene
Chlorogenic acid
Chediak extract

Formulation of Pharmaceuticals for the Diabetic

I. Preliminary Studies in the Induction of Alloxan Diabetes in Rabbits*,†,‡

By GORDON B. STIRLAND and GEORGE E. CROSSEN

In a study of the use of alcohol, glycerin, and propylene glycol in pharmaceuticals for diabetics, it was found that alcohol given in four divided doses of 0.45 cc./Kg. each at one-hour intervals did not raise the blood sugar to any greater extent than did water given in a similar manner. Fluctuation in the blood sugar curves of the animals, caused by excitation, prevented accurate determination of the effects of alcohol and water and could not be satisfactorily overcome by anesthesia with pentobarbital sodium or by partial anesthesia with urethane. Morphine showed more promising results, indicating a need for further study.

CONFLICTING REPORTS in the literature (1–6) raise some question as to whether alcohol and certain other pharmaceutical vehicles are glycogenic and thus whether their use in medicinals for diabetics is justified.

Examination of these reports reveals that many different methods and many experimental subjects, differing both in quantity and type, have been employed to test the effect of these vehicles on the concentration of blood sugar in both normal and diabetic animals.

The present study was undertaken in the attempt to set up a standardized procedure by which the effects of alcohol and other possible glycogenic materials could be observed in alloxan-induced diabetic animals. Such a procedure, if applied in the proper statistical manner to a number of animals, should yield results upon which statistically sound and scientifically usable conclusions can be based.

EXPERIMENTAL

The animals used in these experiments were New Zealand white rabbits. They were fed commercial rabbit pellets in amounts sufficient to supply the minimum daily food requirements, since diet has been shown to be a factor in alloxan diabetes (7). Food was withheld for twenty-four hours prior to alloxan administration and twenty-four hours prior to each series of experiments with the liquids to be tested.

Blood sugar was determined by the micro method of Jeghers and Myers (8) which lent itself well to this study because of the small quantity of blood required for each determination. A Cenco colorimeter with a green filter was employed for the colorimetric readings.

Alcohol solution (50%) was administered in four divided doses. The quantity given in each dose was calculated as the amount received by an individual of 70 Kg. body weight taking a 50% alcoholic medicine in doses of 5 cc. every hour for twelve hours, or 0.45 cc./Kg. per day. The results of the administration of this amount of alcohol were compared with those obtained with a similar quantity of water, using an equal number of animals. Blood sugar determinations were made once each hour, each sample being taken just prior to administering the test material.

A holder modified somewhat from that pictured in Remington (9) was used to secure the animals. After a blood sample was obtained, the holder was raised to an almost vertical position, a catheter was inserted into the stomach, and the liquid was introduced directly. The rabbits were handled in as uniform a manner as possible.

Results from experiments with normal animals showed a good deal of fluctuation in blood sugar levels, despite measures taken to overcome this. In a single experiment with four normal animals (Fig. 1), the emotional response to the experimental procedure alone was found to cause sufficient variance in blood sugar levels to dissemble the effect of the liquid tested.

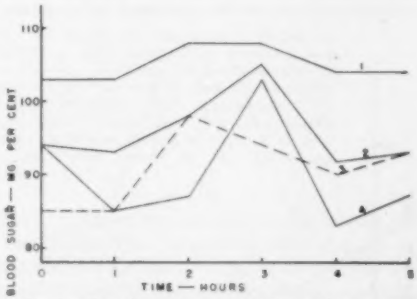

Fig. 1.—Blood sugar concentrations of four normal animals. Effects of experimental procedure. Catheter alone to 1 and 4; catheter plus water to 2 and 3.

To induce diabetes, 200 mg./Kg. of a 5% solution of alloxan monohydrate was injected intravenously at fifteen-minute intervals (10). Triphasic blood sugar curves were obtained following the alloxan injections. To combat the hypoglycemia, 5%

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Presented to the Scientific Section, A. PH. A., Buffalo meeting, August, 1951.
† Submitted to the Graduate School of the Oregon State College by G. B. Stirland in partial fulfillment of the requirements for the degree of Master of Science.
‡ This work was supported by a grant from the American Foundation for Pharmaceutical Education.

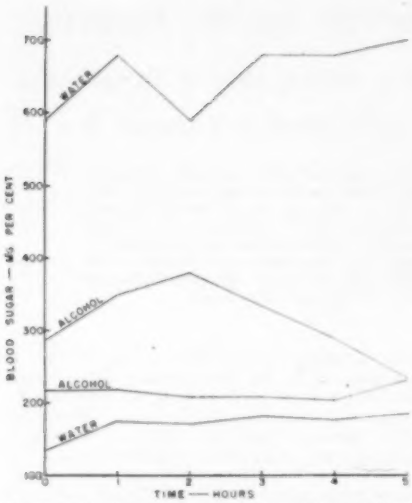

Fig. 2.—Blood sugar concentrations of four diabetic animals. Effect of administration of alcohol.

dextrose was given intravenously and 10% orally where required.

In the first experiment with diabetic animals, fluctuations similar to those seen with normal animals occurred in the blood sugar readings. This effect is shown in Fig. 2. To determine whether normal animals respond to this experimental treatment in the same manner as diabetic animals, a study was conducted in which a series of treatments was given to 6 normal animals which were then rendered diabetic and subjected to the same procedure. The results of this experiment are shown graphically in Figs. 3–6.

An attempt was made to overcome the excitation factor present in these studies. Anesthesia with pentobarbital and partial anesthesia with urethan

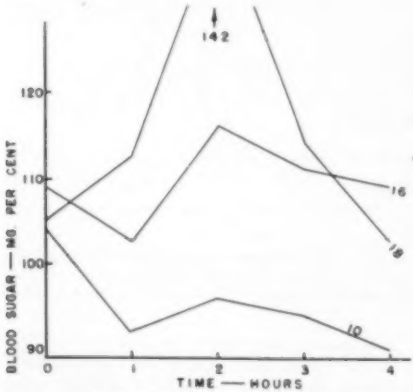

Fig. 3.—Blood sugar concentrations of three normal animals. Effect of administration of water.

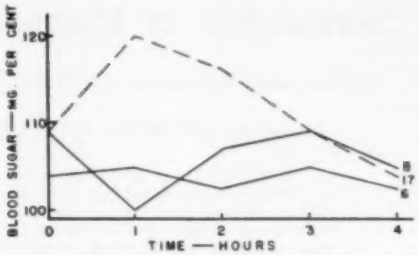

Fig. 4.—Blood sugar concentrations of three normal animals. Effect of administration of alcohol.

were attempted without success; however, 10 mg. of morphine injected intraperitoneally produced more promising results.

SUMMARY AND CONCLUSIONS

Results obtained in this study indicate that alcohol, in the amount of 0.45 cc./Kg., does not influence blood sugar concentration in either normal or diabetic rabbits to any greater extent than does water. Sudden rises in the level of blood sugar noted after administration of alcohol are also noted following administration of water. This was attributed to the emotional response of the animal, which made it difficult to ascertain the effect of the material administered. No apparent allowance has been made in previous studies for this important factor; hence, there is some doubt as to the reliability of the conclusions contained in these reports.

Fluctuations attributed to emotional response in the diabetic rabbits were of a greater magnitude than those in the normal animals since the blood sugar level in diabetics is not controlled to the same extent as in normal animals. Also, there is some transfer of response from the physical experience of the alloxan injections, evidenced by the animal's greater reluctance to be secured in the holder and by an increased sensitivity to handling. These responses were manifested when the animal was utilized in further experiments following the injections.

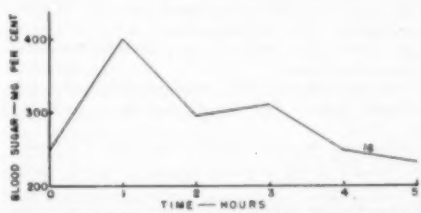

Fig. 5.—Blood sugar concentration of one diabetic animal. Effect of administration of water.

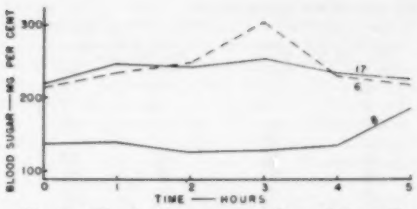

Fig. 6.—Blood sugar concentrations of three diabetic animals. Effect of administration of alcohol. (Compare with Fig. 4.)

The method used here for induction of diabetes in rabbits was found to be reliable; the greatest difficulty arose in the loss of some animals during unsupervised periods. Likewise, the other methods employed in this study could be applied to a standardized procedure if the exceptions noted could be overcome.

The use of modern statistical methods has become increasingly important in biological experimentation. In studies of this type, it is evident to statisticians that much of the disagreement between previously cited results of the effect of alcohol on blood sugar concentration arises from the lack of application of statistical methods (e. g., randomization, sample size) in those studies. Application of such methods (11) to the present study indicates that from 50 to 100 rabbits must be used to produce results which are statistically reliable; the actual number of subjects depends upon the degree of accuracy desired. Further studies with morphine or other drugs which act in a similar manner might reduce the variance in a group of animals, such as were used here, with a resulting reduction in necessary sample size.

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Xanthine Oxidase Studies*,†

By SANFORD O. BYERS‡

An investigation was undertaken of the effect on the purine metabolism of the rat of 2-amino-4-hydroxy-6-formyl pteridine, known to inhibit xanthine oxidase, and sodium bromide, reported to retard uricopoiesis. Neither the intraperitoneal administration of the pteridine nor the oral administration of sodium bromide significantly lowered the tissue concentration of "soluble urate" or prevented the further appearance of such urate upon incubation of the tissues. No comparable inhibition of the *in vitro* activity of xanthine oxidase was shown by more than 300 other compounds, many of similar structure, when tested by the Thunberg technique.

THE COMPOUND 2-amino-4-hydroxy-6-formyl pteridine, as reported by Kalckar, *et al.* (1), is a powerful inhibitor of xanthine oxidase *in vitro*. Since the *in vivo* inhibition of xanthine oxidase could lead to a better understanding of purine metabolism and perhaps to a drug useful in the treatment of gout, the action of this pteridine on the purine metabolism of the rat was investigated. A similar trial was made of the effect of sodium bromide, since bromine has been reported (2) to retard uricopoiesis. In addition, over 300 compounds including 35 pteridines, were tested for their ability to inhibit crude milk xanthine oxidase. Of all the compounds, only the 2-amino-4-hydroxy-6-formyl pteridine showed significant inhibition, and even this substance, active *in vitro* in high dilution, was without significant effect when injected intraperitoneally in rats.

* Received June 5, 1952, from the Mount Zion Hospital, Harold Brunn Institute, San Francisco, Calif.
† This work was aided by grants from the California Wine Institute and the U. S. Public Health Service.
‡ The author wishes to express thanks to Mary Alice Garms for valuable technical assistance.

TABLE I.—TISSUE URATE BEFORE AND AFTER INCUBATION

Tissue	Untreated Rats, Mg./100 Wet Wt.%,[b] Before	After	Pterin-Injected Rats, Mg./100 Wet Wt.%,[c] Before	After	Oral NaBr Rats, Mg./100 Wet Wt.%,[d] Before	After
Brain	3	4	2	3	3	4
Skeletal muscle	6	7	4	5	5	5
Testicle	17	55	9	21	12	15
Lung	17	71	18	42	..	..
Spleen	30	101	25	76	47	99
Kidney cortex	26	89	26	110	32	91
Kidney medulla	23	67	18	94	19	63
Liver	8	6	3	2	2	2

[a] Each experiment incorporates analyses of like tissues pooled from 3 rats. Tissues were incubated for two hours at 37°
[b] Averages from 6 experiments.
[c] Averages from 2 experiments.
[d] Results of one experiment.

METHODS

The pteridine was suspended in 2 Gm. % concentration in a 1:1 mixture of benzyl benzoate and corn oil. A single intraperitoneal injection averaging 40 mg. was given to each of six male albino rats, 180–200 Gm. in weight. Four days later the animals were killed and tissues were analyzed for both content and rate of production of "soluble urate," as described elsewhere (3). Urine was collected during the twenty-four hours immediately preceding sacrifice and analyzed for allantoin according to published methods (4). During the first forty-eight hours after injection, the animals were maintained on Purina Laboratory Chow and water. During the last forty-eight hours, they were allowed only 5% glucose *ad lib.* so as to eliminate any influence of purine ingestion and to provide a maximal urine flow for collection. Controls were treated similarly but were not injected.

Sodium bromide was administered for twenty days to each of 4 male albino rats as a 225 mg. % solution in the drinking water. The animals were fed Chow for eighteen days. During the last forty-eight hours, they subsisted on glucose added to the bromide water to make 5 Gm. %. Urine was collected during the twenty-four hours preceding sacrifice, and urine and tissue analyses were performed as above.

The inhibitory effect of the 2-amino-4-hydroxy-6-formyl pteridine and other compounds was tested *in vitro* by following the rate of reduction of methylene blue by milk xanthine oxidase acting on a sodium xanthate substrate according to the Thunberg technique (5). This method was chosen over manometric or spectrophotometric methods because its comparative rapidity permitted the survey of a large number of compounds.

The reaction was arranged by placing in the bottom of the Thunberg tube 2.0 ml. of $^1/_{15}$ *M* phosphate buffer of *p*H 7.0, 0.5 ml. of 0.005% methylene blue chloride, 0.3 mg. of 0.05 *M* sodium xanthate, inhibitor solution, and distilled water sufficient to make the volume to 4 ml. One-half milliliter of homogenized, pasteurized milk was placed in the sidearm. A control, consisting of a tube in which boiled milk and 0.05 ml. of methylene blue chloride were substituted for fresh milk and the usual amount of methylene blue, was run in each analysis to serve as a visual standard for 90% reduction. All tubes were evacuated for three minutes in a manifold connected to a HyVac pump, each tube being tapped and shaken 4 times in this interval. They were then equilibrated for one-half hour in an air incubator at 37° before the enzyme was tipped in. The reaction was allowed to proceed at 37° and the time required for the color to fade to that of the 90% reduction standard was noted.

RESULTS AND DISCUSSION

The average twenty-four hour output of allantoin in the urine of the 6 rats receiving 2-amino-4-hydroxy-6-formyl pteridine was 31 mg.; the corresponding average of 4 rats drinking bromide was 32 mg.; and the comparative figure for normal rats, as previously published (6), was 34 mg. Since allantoin represents the excretory end product of purine nitrogen in this species, no inhibition of purine metabolism was evident.

The data in Table I show the average content of "soluble urate" in various tissues from normal rats, from animals which had received the amino-hydroxy-formyl pteridine, and from rats which had received sodium bromide. The additional urate produced by each tissue during a two-hour incubation period is also shown. It is seen that all tissues normally showing an increase in "soluble urate" after incubation also show this capability when derived from treated rats. The increase for testicle is small in the pterin-injected rats, but at the lower end of the normal range. No significant increase is shown for testicle in the single experiment on pooled tissue from rats receiving bromide. The values for liver before incubation in the experimental animals are also in the low normal range.

The 2-amino-4-hydroxy-6-formyl pteridine strongly inhibited the *in vitro* xanthine oxidase reaction in 10^{-5} molar concentration; other substances were tested in concentrations of 0.001 *M* and higher, but none had an effect comparable to the pteridine.

Each substance was tested at first in concentrated or saturated solution. If the time for decolorization in such concentrated inhibitor solution was six minutes or more (compared with a control value of three minutes), the substance was retested at a concentration of 0.002 *M*. Only folic acid and 2-amino-4-hydroxy-6-formyl pteridine were active in this range. The activity of folic acid was presumably caused by the presence of the latter pteridine as impurity. The pteridine itself, in concentration of 10^{-5} molar, was capable of preventing decolorization for sixteen hours. Among the sub-

stances tested were: 35 pteridines,[1] including 2-amino-4-hydroxy pteridine, 2,4-diaminopteridine, the 2-amino-4-hydroxy-6-hydroxymethyl compound, the 2-amino-4-hydroxy-6-pteridine carboxylic acid; some other pteridines tested include the 2-thiol-4-amino-6,7-dihydroxy, the 2-thiol-4,6,7-trihydroxy, the 2-thiol-4-amino, the 2-thiol-4,7-dihydroxy-6-methyl, the 2-amino-4,6,7-trihydroxy, the 2,4-diamino-6,7-dihydroxy, and the 2,4-diamino-6-hydroxy-7-methyl; 9 straight-chain urea or guanidine compounds, including urea oxalate, biuret, acetylmethylurea, aminoguanidine bicarbonate, phenylurea, 1,3-diethylurea, and *p*-ethoxy aniline amide; 16 barbituric acid derivatives and 6 other cyclic urea compounds including allantoin, alloxan, alloxantin, and parabanic acid; violuric acid, diphenylhydantoin, barbital, amytal, and 5-nitro-barbiturate; salicylates, benzoic acid, benzilic acid,[2] and over 40 halogen, nitro, and amino derivatives of these compounds, including acetylsalicylic acid, salicylaldoxime, 5-nitrosalicylate, 3,5-dinitro-salicylate, diiodosalicylate, salicylaldehyde, thio-salicylate, *p*-hydroxy benzoate, *p*-fluorobenzoate, *p*-chlorobenzoate, 2-chloro-5-nitrobenzoate, 3,5-diiodo-4-hydroxybenzoate, 2-hydroxy-5-chlorobenzaldehyde, *o*-hydroxybenzyl alcohol, orthoform, and 2,4-dihydroxybenzophenone; 7-phthalimide, phthalylhydrazide, and thiazole derivatives, including 3-aminophthalimide, 4-aminophthalylhydrazide, *cis*-Δ^4-tetrahydrophthalic anhydride, and 1,4-phthalazione; 5-benzimidazoles and benzothiazoles,[3] including 6-nitro-2-chloro-benzothiazole and 5-methylbenzimidazole; several purines, including uric acid, theobromine, and caffeine; some pyrones, including kojic acid; quinolines, including 3-aminoquinoline, oxyquinoline sulfate, tetrahydroxyquinone, quinine, quinidine, phenyl quinaldyl ketone, *p*-dimethylaminophenyl-6-ethoxyquinaldyl carbinol, and *p*-dimethylaminophenyl-6-methoxyquinaldyl ketimine; furyl and quinolyl derivatives of acrylic and cinnamic acid[4] including β-(2-thionyl)acrylic acid, β-(3-indole)acrylic acid, β-(4-quinolyl)acrylic acid, β-(2-furyl)acrylic acid, *p*-hydroxy cinnamic acid, *p*-diethylaminocinnamic acid, β-(2-furyl)-acrylic acid, *p*-hydroxycinnamic acid, and *p*-diethyl-aminocinnamic acid; and other compounds showing biological activity such as cincophen, cinchonine, and rotenone, including some antibiotics such as sulfanilamide derivatives, streptomycin, penicillin G, and aureomycin; vitamins including rutin, inositol, pyridoxine, B_{12}, and thiamine; and narcotics such as scopolamine and phenacaine.

Kalckar, Kjeldgaard, and Klenow (1) mention the existence of enzymes in milk and liver which may destroy inhibitors of xanthine oxidase. Those of the compounds listed above which tested negatively *in vitro* were tested in high concentration or in saturated solution, frequently with excess solid present. It is unlikely that inactivating enzymes would be so powerful as to prevent any signs of inhibitory action from appearing. On the other hand, a 10^{-3} molar solution of pteridine was sufficient to block the action of xanthine oxidase for sixteen hours. This also argues against the presence of significant enzymic obstruction in the pasteurized milk. It is of course not altogether impossible that enzymes might be present which would inactivate pteridines closely related to the 2-amino-4-hydroxy-6-formyl compound, even when these pteridines are present in excess of their solubility limits.

The failure of the 6-formyl pteridine to be active *in vivo*, although it is highly active *in vitro*, is unexplained. Although an apparent possible explanation is the presence of liver enzymes destructive of 6-formylpteridine, this does not seem actually to be the case. Thus Dietrich, Monson, Williams, and Elvehjem (7) also found the 6-formyl pteridine highly active against xanthine oxidase activity in chick liver homogenate, but quite inactive *in vivo*.

[1] Pteridines were very kindly supplied by Dr. Emory Gal of the University of California at Berkeley, by Dr. C. W. Waller of Lederle Laboratories, and by Dr. C. K. Cain of Cornell University.

[2] Benzilic acid derivatives were furnished by the Heyden Chemical Corporation, Cu-3-phenyl salicylate by Dow Chemical Co.

[3] Obtained through the courtesy of Dr. Frederick French of the Department of Chemotherapy, Mount Zion Hospital.

[4] We are grateful to Dr. Arthur Furst of Stanford University for providing these compounds.

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Tolerance, Toxicity, and Caloric Availability of Intravenously Injected Tapioca Dextrin Solution in Rabbits*,†

By LAWRENCE E. DETRICK, ALVIN E. LEWIS, DOROTHY N. HIGHBY, BONNIE RHODES, and THOMAS J. HALEY‡

Dutch strain rabbits were given eleven consecutive daily intravenous injections (via an indwelling plastic catheter) of tapioca dextrin solution containing amino acid hydrolysate, B-complex vitamins, and adequate potentially available calories for complete parenteral nutrition. Intravenous tapioca dextrin was shown not to be a source of high caloric intake. The intravenous regime was well tolerated by the majority of animals. Recovery occurred in those animals developing symptoms of toxicity if the infusions were not continued too long after their onset. Hydropic degeneration found in the epithelium of the proximal convoluted tubules of the kidney at the end of the injections was reversible during an oral feeding recovery period. Hyaline degeneration was not observed in the glomeruli of the kidney or the blood vessel in the lungs, and foam cells were absent in the reticulo-endothleial system.

COMPLETE parenteral nutrition is limited by the lack of suitable means for providing an adequate caloric intake (1). Isotonic glucose intravenously administered for complete caloric requirement is accompanied by a fluid intake which is prohibitive, and hypertonic glucose scleroses vessels. Many carbohydrates have been tried as an intravenous source of calories but are not utilized in quantity unless previously reduced to their basic glucose units (2). Rice, *et al.* (3) have devised a parenteral solution containing invert sugar, amino acids, ethanol, and water which has 1000 calories per liter.

Dextran, a polysaccharide, labeled with C^{14} and injected intravenously, has been reported to be subject to complete oxidation in the mouse (4). Engstrand and Åberg (5) have found 28 per cent of the intravenously infused dextran to be excreted in the alimentary canal of animals in nine hours, but the ultimate fate of dextran and other plasma substitutes remains to be determined (6).

Intravenous fat emulsions have been administered for their high caloric value, but the problems associated with the production of a suitable stable emulsion which will withstand the rigors of rough handling, temperature changes, and storage remain to be solved (7).

Preliminary studies made elsewhere indicated that macromolecular tapioca dextrin, given intravenously to rabbits or intraperitoneally to rats, provided an adequate source of carbohydrate (8). That investigation compared the utilization of intravenous 36 per cent tapioca dextrin with that of 38.5 per cent glucose in rabbits; when calculated as glucose, from 13 to 17 per cent of the former and 46 per cent of the latter solution appeared in the urine in twenty-four hours. Rats on an inadequate basal diet received intraperitoneal injections of a 35 per cent tapioca dextrin solution and derived a body weight benefit from the injections. However, when used clinically, tapioca dextrin appeared in the urine faster than it could be utilized by the body.

Inasmuch as there were no data available on the effect of continuous intravenous administration of tapioca dextrin on the body weight curve or on its acute and chronic toxic effects, these factors have been investigated, since such information is necessary before more extensive clinical trials with tapioca dextrin can be undertaken.

EXPERIMENTAL

Adult male and female Dutch rabbits (average weight, 2,100 Gm.) were given 11 daily intravenous infusions (Table I) via an indwelling polyethylene catheter (9). The daily injection of R4 solution (see Table I) supplied an adequate potentially-available caloric intake, but no change was made in the daily dosage to compensate for subsequent body weight losses. Half of the animals were sacrificed at the end of the injection period. Their tissues were examined grossly, and samples of the kidney, spleen, liver, lung, pancreas, adrenal gland, and muscle were taken for histological study. Sections were stained routinely with hematoxylin and eosin, but liver and kidney slices were also fixed in absolute alcohol and studied for glycogen with Best's carmine stain. The remainder of the animals

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‡ The authors wish to thank Dr. Melvin Schoenberg for his molecular analysis of tapioca dextrin and to express their appreciation to Don Baxter, Inc., of Glendale, Calif., for help in the preparation of the parenteral solutions.

TABLE I.—COMPONENTS AND CHARACTERISTICS OF PARENTERAL SOLUTIONS R1 TO R6

	Animal Group		
	A	B	C
Infusion solution	R1–R4	R5	R6
Tapioca dextrin, % (w/v)	30	0	0
Casein hydrolysate, % (w/v)	4.3	4.3	4.3
Thiamin, % (w/v)	0.005	0.005	0.005
Riboflavin, % (w/v)	0.01	0.01	0.01
Nicotinamide, % (w/v)	0.05	0.05	0.05
Glucose (anhyd.), % (w/v)	0	0	10
Tonicity[a]	11.5	5.0	14.0
Calories per 150 cc.	216	26	82

[a] Tonicity is expressed in terms of anhydrous glucose solution. A tonicity of 11.5 is equivalent in tonicity to an 11.5% anhydrous glucose solution. Since a 5.3% solution of anhydrous glucose is isotonic with blood, solution R4 is hypertonic and has an osmotic pressure of approximately 2.1 times that of blood.

were allowed a recovery period, given the regular colony diet of Albers Green rabbit pellets, greens, and high protein pellets (Albers Calf Manna), then were sacrificed and similarly studied for tissue changes. The body weight was observed daily as a manifestation of the caloric availability of the intravenously administered nutrient solution. The animals' physical condition, alertness, general activity, and body weight curves were correlated daily as an expression of the over-all nutritional and chronic toxic effects of the experimental procedure.

Source and Chemical and Physical Properties of 36% Tapioca Dextrin Solution (8).—Tapioca dextrin is obtained by acid hydrolysis of a starch derived from the root of the cassava plant. The starch has been reported to contain approximately 80% amylopectin (highly branched polysaccharide of glucose units) and 20% amylose (linear polysaccharide of glucose units). The chemical constituents and the physical properties of 36% tapioca dextrin solution are tabulated in Tables II and III, respectively.

A 36% parenteral tapioca dextrin solution is a clear, amber, sterile, nonpyrogenic, slightly viscous solution suitable for intravenous injection. Physical characteristics resulting from a reduction of tapioca dextrin from 36–30% and the inclusion of amino acids and vitamins were not determined. Specifications for the components, viscosity characteristics, and caloric value of parenteral solutions R1 to R6 are given in Table I. All solutions were infused at a *p*H of 6. Animal groups A, B, and C refer to groups of animals which received intravenous feeding and whose average body weight curves are illustrated as curves *A*, *B*, and *C*, respectively, in Fig. 1.

TABLE II.—CHEMICAL CONSTITUENTS OF 36% TAPIOCA DEXTRIN SOLUTION

	Gm./100
Solids	36.00
Ash	0.06
Chloride	0.02
Sulfate, 0.01 *M*	0.004
	P. P. M.
Potassium	220
Calcium	100
Phosphorus	45
Magnesium	39
Sodium	5–10
Silicon	5–10
Iron	5–10
Boron	0–5
Aluminum	0–5
Copper	0–5
Strontium	0–5
Lead	0–1
Manganese	0–1
Nickel	0–1
Chromium	0–1

RESULTS

Acute Toxicity Due to High Dosage Rate.—The potential toxic effect of a high intravenous dosage rate became apparent almost immediately. Daily infusions of 150 cc. of R1 solution at a rate of 50 cc./hr. resulted in death on the ninth day. The body weight curve fell steadily for five days (similar to curve *A*, Fig. 1); the general appearance, alertness, and activity of the animal were normal. On the sixth and seventh days the weight curve deviated to the right (flattened out and slightly elevated), then rose precipitously on the eighth and ninth days almost reaching its initial level, and was accompanied by progressive physical deterioration. By the ninth day the animal was almost too weak to stand. It had an unkempt appearance and the abdomen was distended, tense, and tender. The rabbit appeared in a stupor unless moved or aroused. Moving the animal initiated intermittent tonic and clonic convulsions that progressed until death.

Post mortem examination showed an edematous abdominal skin, muscles, and viscera with an abnormal amount of straw-colored, protein-containing fluid in the pleural and peritonea cavities.

TABLE III.—PHYSICAL PROPERTIES OF 36% TAPIOCA DEXTRIN SOLUTION

*p*H	4.7
Freezing point, ° C.	0.7
Specific gravity	1.12
Rotation spec. $[\alpha]_D^{25}$	180
Spec. cond., micromhos	400
Viscosity, centipoises at 25° C.	14
Titratable acidity, ml. of 1 *N* NaOH/L.	22
Reducing sugars, Gm. glucose/100 ml.	1.5
Nitrogen, Gm./100 ml.	0.004
Apparent mol. wt. of dextrin (freezing point)	2200

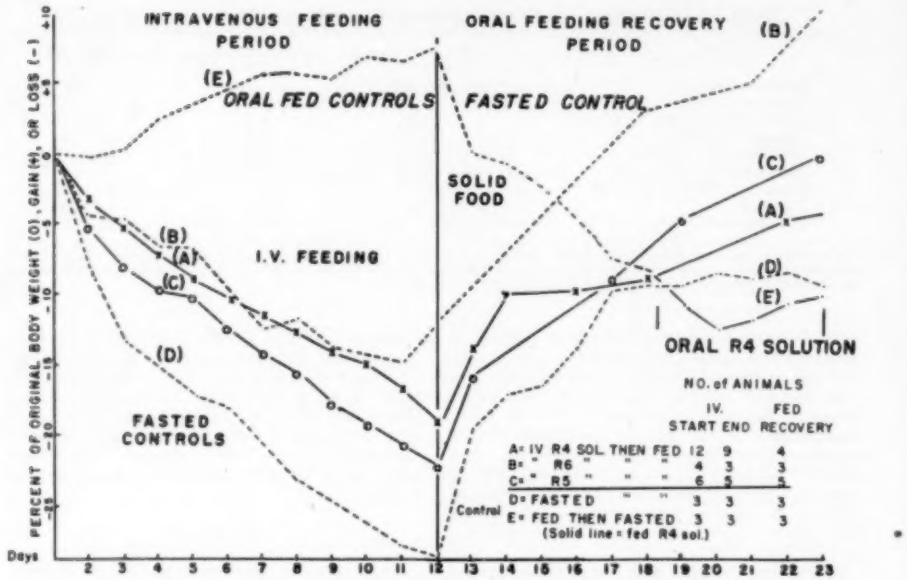

Fig. 1.—Effect of intravenous feeding and recovery on body weight.

Histological sections of the lung and spleen were essentially normal, but the individual liver cells were generally pale and contained small vacuoles. There was a marked generalized swelling and pallor of the tubular epithelium of the kidney. The cytoplasm of these cells was either clear or composed of large confluent empty vacuoles, but the nuclei were intact and neither the collecting ducts nor the glomeruli were involved. The vacuoles in the kidney and liver sections did not contain glycogen.

Tolerance to Lower Dosage Rate.—The majority of normal animals tolerated repeated daily infusions of 150 cc. of R5 solution administered in two 3-hour injection periods with one hour intervening. Tolerance was accompanied by a steady fall in body weight (without edema) as illustrated by curve *A*, Fig. 1. However, 2 of 12 animals developed the previously described chronic toxic symptoms even when the R5 solution was given at this reduced rate. Recovery occurred if only 3 injections were given after these toxic symptoms appeared, and the animal was placed on the regular colony diet. Under similar conditions, 6 infusions after the onset of toxicity resulted in death of the animal five days after the injections were stopped.

Nutritional Value of Intravenously Injected Tapioca Dextrin.—The group average percentage of the initial body weight loss, as illustrated in Fig. 1, served as the criterion of caloric availability for prolonged and repeated intravenous infusions of tapioca dextrin or control solutions (Table I).

Of the twelve animals which started (group A, Fig. 1), nine completed the 11 consecutive daily injections of R4 solutions (Table I). Five animals were sacrificed; four were placed on an oral diet during the recovery period and then sacrificed for recovery studies. The body weight of the group A animals steadily decreased throughout the infusion period. A daily injection of 216 potential calories as R4 solution resulted in a group average loss of 19% of the original body weight on the twelfth day of the experiment.

Curve *B*, Fig. 1, shows the effect on body weight of a group of animals similarly injected with a solution (R6) containing 10% anhydrous glucose in place of tapioca dextrin and all other constituents remaining the same (Table I). A daily injection of 82 available calories as R6 solution resulted in body weight curve *B* which does not differ significantly from that of group A. This group of animals was inadvertently fed on the eleventh day of the experiment; therefore, they received only 10 instead of the usual 11 intravenous injections.

Animals represented by curve *C* (Fig. 1) derived their daily exogenous caloric intake (26 calories, Table I) from the amino acids present in parenteral solution R5, since both glucose and dextrin were omitted. The body weight curve for animal group C (−22% on twelfth day) lies significantly below that of group A and above the curve for group D (−30% on the twelfth day), the fasted controls.

An orally-fed group E and a fasted control group D (Fig. 1) which were reversed during the recovery period, were included for comparative purposes.

Histological Change and Reversibility of Tissue Damage.—Kidney changes after 11 tapioca dextrin infusions were similar to, but less intense than, those reported following the onset of the symptoms of toxicity. The remainder of the organs were essentially normal. Foam cells were not observed in the reticulo-endothelial system but liver glycogen

was depleted after 8 injections. Complete reversibility of tissue damage occurred fourteen to twenty-six days after injections were discontinued.

DISCUSSION

Although tapioca dextrin injections were not a high source of calories, the body weight curve was maintained well above the starvation level. Acid hydrolysis used in the preparation of tapioca dextrin liberated reducing sugars that accounted for from 1.8 to 2.25 Gm. of nutritionally available glucose administered daily. The dextrin can be metabolized if taken orally (see curve *E*, Fig. 1). An average of 15 cc. of R4 solution was consumed daily. The favorable weight gain of animal group A over that of fasted group D, Fig. 1, cannot be interpreted as tissue utilization, pending further investigation repeating the technique of Engstrand and Åberg (5), who demonstrated that intravenously injected dextran was excreted into the gastrointestinal tract. Since tapioca dextrin is utilized when given orally, any of the infusion material excreted into the gastrointestinal tract could be digested, reabsorbed, and utilized as glucose. However, from liver glycogen sections, it is known that neither the glucose molecules in the infusion solution nor that postulated as of possible intestinal origin were sufficient to prevent depletion of liver glycogen during the injection period.

Since curve *C* (amino acid control group) is signi..cantly above curve *D* (fasted control group), Fig. 1, it would appear that the amino acid content of solutions R4 and R6 furnished a significant fraction of the body weight gain of curves *A*, *B*, and *C*. This would confirm the observation of Elman (10) that intravenous amino acids are burned for calories when the amount of available carbohydrate is inadequate. The fluid intake was the same for all 3 daily infusion solution dosages. The absence of this hydrophilic property in the latter two solutions (R5 and R6) would increase the tendency for fluid escape into the tissues, thus falsely elevating body weight curves *B* and *C*, Fig. 1.

The small molecular size of the tapioca dextrin particles used (2,200 mol. wt. by freezing point) may be of significance in the tolerance, relative lack of toxicity, and reversibility of the histological changes observed. Spinco analytical ultracentrifuge examination showed tapioca dextrin to be highly polydisperse, in the range of very low molecular weight, probably well below the permeability factor for the glomeruli of the kidney (11). The employment of such low-range molecular weight material precludes the danger of increased toxicity from and reactions to intravenously administered molecular particle sizes above 100,000 mol. wt. (12). On the other hand, in the preparation of Dextran, particles below 40,000 mol. wt. are removed, not because they were observed to enhance the toxic effect, but rather because they were excreted so rapidly in the urine (13). Lippman, Marti, and Oliver (14) suggest a saturation of the tubular absorption capacity by small molecular weight oxypolygelatin to be the principal mechanism responsible for proteinuria, glomerular and tubular damage and azotemia; also they suggest that it might be possible to eliminate the proteinuria and principal toxicity by fractionation to remove the molecules of small molecular size. In the present experiment only small particle-sized tapioca dextrin was used. Eleven consecutive daily intravenous injections of 45 Gm. of this material were tolerated by the majority of animals. During the injection period no untoward effects were observed; the toxic syndrome, previously discussed in connection with injection rates of 50 cc./hr., was lacking; and the hydropic degeneration observed in the epithelium of the proximal tubules of the kidney was reversible. On the basis of reported physico-chemical relationship of macromolecular size to toxicity, reactions, and excretions, it is suggested that the small size of the tapioca dextrin molecule *per se* is of significance in the results reported.

Histological changes observed at the end of the period of injections, but which were reversible during the recovery period, appear comparatively mild to those reported by Hartman (15) for other intravenously injected macromolecular substances given in smaller doses but as larger particle sizes. In the present study, massive foam cell formation in the reticulo-endothelial system or a hyaline degeneration involving the glomeruli of the kidney or the blood vessel walls in the lungs could not be demonstrated.

CONCLUSIONS

1. An intravenously injected tapioca dextrin solution is not a source of high caloric intake.

2. A parenteral solution of tapioca dextrin, amino acid hydrolysate, and vitamins, when intravenously injected at a rate of 25 cc. per hour, in two 3-hour injection periods with a one-hour rest period intervening, and in daily doses of 150 cc., has been shown to be well tolerated by the majority of normal rabbits for eleven consecutive days.

3. This histological kidney damage observed after 11 daily injections of parenteral tapioca dextrin has been found to be reversible during the oral feeding recovery period.

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Chemical Constitution and Analgetic Action*,†

By M. B. SLOMKA‡,§ and F. W. SCHUELER

The relation between chemical constitution and analgetic action has been investigated in several compound types that bear a structural relationship to the morphine-type analgetics. Some degree of analgetic potency was demonstrated in several compounds. Some aspects of the relationship between chemical constitution and analgetic action are discussed.

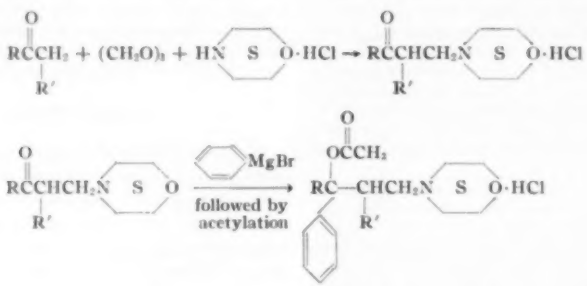
THE immense literature concerning constitutional studies of the morphine molecule and the morphine-type analgetics reflects both an industrial and academic interest in structure action relationship. Lee (1) and Eddy (2) have summarized the few attempts to formulate the constitutional requirements for analgetic action and the effects of constitutional changes on analgetic potency within the several analgetic series.

Schueler, Gross, and Holland (3) noted the "presence of both sympathomimetic and parasympathomimetic-like moieties connected (in general) by the same amino nitrogen atom" in the structure of morphine and its analogs, and further suggested that the analgetic action of these agents may be traceable to the presence of such moieties within the same molecule. Slomka and Schueler (4) prepared 11 compounds containing the aforementioned moieties and evaluated their analgetic potency as a test of this working hypothesis. The outcome warranted a continuation of the investigation, and the results of this further study are described in the present report.[1]

The structures of all the compounds prepared to test the hypothesis of Schueler, *et al.*, are shown in Table I.

EXPERIMENTAL

Synthetic Procedures.—(*a*) 1,1-Diphenyl-3-morpholino-propyl acetate hydrochloride, 1-acetoxy-1-phenyl-2-morpholinomethyl-cyclohexane hydrochloride, and 1-methyl-1-phenyl-3-morpholino-propyl acetate hydrochloride were prepared as follows:



The corresponding β-morpholinoethyl compounds were prepared in the manner previously described by Harradence and Lions (6). The β-morpholinoethyl ketone (0.12 mole) was added to an ether solution of phenyl magnesium bromide prepared from 0.5 mole of magnesium and 0.25 mole of bromobenzene under anhydrous conditions. On completion of the addition, the mixture was refluxed for one hour, poured over ice, and made alkaline to litmus with cold 20% sodium hydroxide solution. The tertiary β-morpholinoethyl alcohol was extracted with ether; the ether solution was dried over sodium hydroxide sticks and filtered, and an excess of acetyl chloride was added. A white precipitate separated out in 40% yield, and was recrystallized from alcohol and ether until of constant melting point.

During attempts to purify the hydrochloride of 1-phenyl-1-acetoxy-2-morpholinomethyl-cyclohexane, a separation was made into a low-melting form (A) and a high melting form (B). Since the chloride content for both forms agrees with the theoretical content for the compound, A and B may represent two of the four possible optical isomers.

(*b*) N-(acetonyl-methyl)-ephedrine hydrochloride and N-(phenacylmethyl)-ephedrine hydrochloride were prepared as follows: 60 cc. of acetone or 120 cc. of acetophenone, 68.5 Gm. of ephedrine hydrochloride, and 16.8 Gm. of trioxymethylene were mixed in 100 cc. of absolute alcohol, and the

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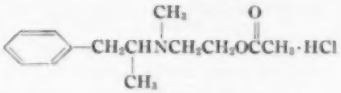
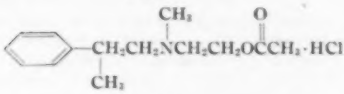
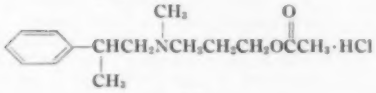
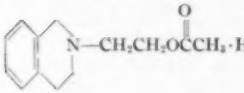
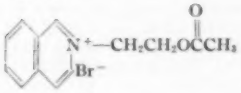
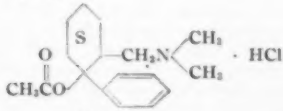
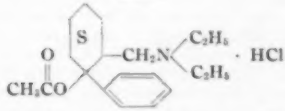
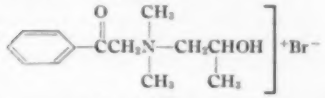
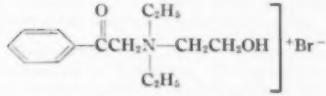
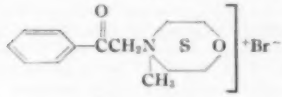
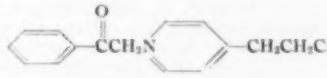
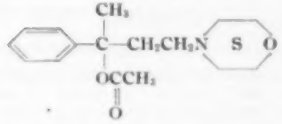
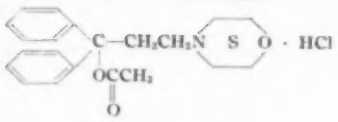
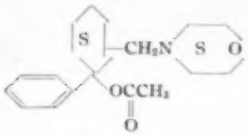
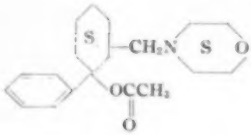
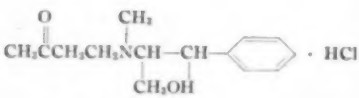
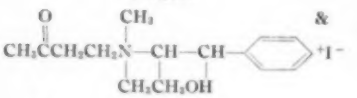
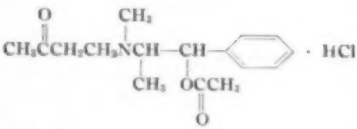
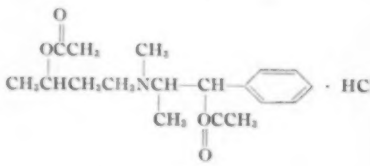
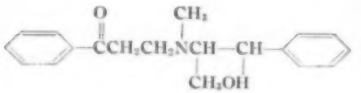
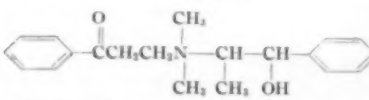
TABLE I.—COMPOUND STRUCTURES AND PHYSICAL CONSTANTS

Compound No.	Formula	Chloride Calcd., %	Chloride Anal., %[a]	M. P., °C.[b]
1[c]		...	...	...
2[c]		...	...	...
3[c]		...	...	...
4[c]		...	...	...
5[c]		...	...	...
6[d]		...	...	...
7[c]		...	...	...
8[c]		...	...	...
9[c]		...	...	...
10[c]		...	...	...
11[c]		...	...	...
12[d]		9.45	8.66	239–241

(*Continued on p. 620*)

TABLE I.—(*Continued from p. 619*)

Compound No.	Formula	Chloride Calcd., %	Chloride Anal., %[a]	M. P., °C[b]
13[d]		11.30	10.74	200
14A[d]		10.05	10.56	200–201
14B[d]		10.05	10.66	254–256
15[d]		13.04	13.06	133–134
16[d]		...	...	214–215
17[d]		12.35	11.30	137–138
18[d]		9.91	10.45	237–238
19[d]		10.62	10.11	223–224
20[d]		...	...	212–213

[a] Chloride analysis by a microquantitative modification of the method of Kanz and Pöhm (5).
[b] Melting points uncorrected.
[c] The physical constants and results of analgetic tests of compounds 1 to 11 have been reported previously (4). Additional physiologic data for some of these compounds are reported in Table III.
[d] The results of analgetic tests of compounds 12 to 20 are reported in Table II. Additional physiologic data for some of these compounds are reported in Table III.

mixture was refluxed for ten hours. The alcohol and the excess ketone were distilled off under reduced pressure, and the remaining oily liquid was taken up in absolute alcohol and precipi-

tated by ether as an oily solid. On recrystallization from acetone, a white solid was obtained which repeatedly recrystallized from alcohol and ether to a constant melting point; yield, 30%.

Both compounds gave a negative result when subjected to the identity test for ephedrine hydrochloride (7).

The corresponding methiodides were prepared by treating an ethereal solution of the above tertiary amino ketones with excess methyl iodide.

(*c*) N-Acetonylmethyl-O-acetyl ephedrine hydrochloride was prepared as follows: a dry ethereal solution of N-acetonylmethyl ephedrine was treated with an equimolecular amount of acetyl chloride and an equal volume of acetic anhydride. The mixture was warmed on a hot plate for two hours and allowed to stand tightly stoppered overnight. The excess reagents were removed *in vacuo*, and the oily residue was recrystallized from a minimum amount of acetone to a white crystalline solid, which was recrystallized from acetone to constant melting point; yield, 20%.

(*d*) N-(3-acetoxy-butyl)-O-acetyl ephedrine hydrochloride was prepared as follows: 5 Gm. of N-acetonylmethyl ephedrine hydrochloride, suspended in 100 cc. of tetrahydrofuran, was gradually added over a thirty-minute period to 0.4 Gm. of lithium aluminum hydride suspended in 100 cc. of tetrahydrofuran. An additional 0.05 Gm. of lithium aluminum hydride was then added, and the mixture was warmed on a hot plate for thirty minutes. After cooling, the excess lithium aluminum hydride was decomposed with acetone, and 20% sodium hydroxide solution was added until a marked separation into 2 layers occurred, at which time the aqueous layer was alkaline to litmus. The tetrahydrofuran layer was removed, and the aqueous magma was extracted twice with 50-cc. portions of ether which were combined with the tetrahydrofuran solution; the whole was then concentrated to a volume of 50 cc. The addition of acetyl chloride resulted in the precipitation of a white solid which was repeatedly crystallized from alcohol and ether until of constant melting point.

Table I summarizes data concerning melting points and analyses of the compounds synthesized.

Analgetic Tests.—The data from the analgetic tests are summarized in Table II. The analgetic activities of the compounds were determined on Sprague-Dawley strain albino rats, weighing from 80–140 Gm., seventy minutes after intraperitoneal administration of the compound in physiological saline solution, using a modification of the D'Amour and Smith technique (8). The tails of the rats were blackened by dipping in India ink. The number of animals used at each dose level is noted. The Null hypothesis and Student's "*t*" were used to determine the significance of the results.

Circulatory System Studies.—Cats were anesthetized with 0.75 ml./Kg. of Dial-urethane (Ciba) intraperitoneally. The right common carotid was cannulated and the blood pressure was recorded on a smoked drum by means of a mercury manometer. Drugs in physiological saline were injected into a femoral vein. Results of these studies are summarized in Table III.

Isolated Gut Studies.—Strips of rabbit ileum, of approximately 2.5 cm. in length, were suspended in oxygenated Tyrode's solution in an isolated tissue bath maintained at 37.8°. After a constant amplitude of spontaneous contractions was reached, the drugs were added to the bath dissolved in Tyrode's solution. Results of these studies are summarized in Table III.

TABLE II.—ANALGETIC TESTS

Compound No.	Mean % Increase in Time for Tail Twitch	Confidence Limit, %	Dose, Mg./Kg.	No. of Animals
12	26.5	5–2	50	10
	38.0	1–0.1	100	10
13	inactive	...	50	10
	inactive	...	100	10
14A	inactive	...	50	9
	inactive	...	100	9
14B	inactive	...	50	10
	inactive	...	100	10
15	inactive	...	50	10
	inactive	...	100	10
16	inactive	...	50	10
	inactive	...	100	10
17	34.7	20–10	50	10
	104.0	0.1	100	16
18	145.0	0.1	100	4
19	inactive	...	50	10
	inactive	...	100	10
20	inactive	...	50	10
	inactive	...	100	10
Morphine Sulfate	72.5	0.1	3	13
Morphine Sulfate	150.0	0.1	4	10
Morphine Sulfate	100.0	...	3.3[a]	
Morphine Sulfate	110.0	0.1	3.3	20

[a] Calculated from log dose-response curve.

DISCUSSION

Of the compounds tested, only 3 demonstrated analgetic activity with the D'Amour and Smith technique. The analgetic action of compound 7 is lost when the diethylamino group is cyclized in the form of a morpholine ring (compound 14). However, opening the cyclohexane ring, as in compound 12, apparently results in weak analgetic activity. Substitution of a second phenyl ring, as in compound 13, destroys activity. It would appear that the analgetic activity of these compounds is a result of the interaction between various portions of the molecule rather than an expression of each structural modification.

The analgetic action of ephedrine (3,9) is apparently lost when it is converted to a tertiary amine with either an acetonylmethyl or phenacylmethyl substitution (compounds 15 and 19). The inactivity of the methiodides of these compounds (16 and 20) is expected, since in general the few quaternary derivatives of potent analgetic agents, i. e., morphine, heroin, and codeine, are all less active than the parent compounds. Introduction of one or two acetoxy groups into the former compound provides analgetically active compounds (17 and 18).

TABLE III.—SUMMARY OF EFFECTS OF COMPOUNDS ON THE ISOLATED ILEUM (RABBIT) AND THE CIRCULATORY SYSTEM (CAT)

Compound No.	Isolated Rabbit Ileum Strip		Cat Circulatory System[a]		
	Effect[b]	Drug Concn., Mg./Ml.	Effect on Blood Pressure	Drug Concn., Mg./Kg.	% Change[c]
1	None	10^{-4}	Depressor and pressor	2	− 4 followed by +17
				4	−13 followed by +37
				8	−26 followed by +26
2	Spasmolytic	10^{-3}			
3	Spasmolytic	10^{-4}	Depressor	1	0
				2	−24
				4	−52
4	Cholinergic stimulant (1/1,000 acetylcholine)	10^{-4}	Cholinergic depressor (1/1,000 acetylcholine)	0.5	−14
				1	−33
6	Spasmolytic	10^{-3}			
7	Spasmolytic	10^{-4}	Depressor	1	−13
				1	−31
12	Spasmolytic	10^{-4}	None	2–32	
13	Spasmolytic	10^{-4}			
14A	Spasmolytic	10^{-4}			
14B	Spasmolytic	10^{-4}			
15	Spasmolytic	10^{-3}			
17	Spasmolytic	10^{-4}	Depressor	0.5	0
				1.0	0
				2	− 7
				4	−12
18	Spasmolytic	10^{-4}		8	−30

[a] In each cat, 1 μg. of acetylcholine produced a 33% depression of the blood pressure.
[b] A spasmolytic effect as used herein indicates a marked inhibition or abolition of the spasm produced by 10^{-8} acetylcholine and the increased activity produced by 10^{-5} barium chloride.
[c] The method of calculating the per cent change in blood pressure is illustrated in Fig. 2. A positive sign indicates an increase in blood pressure (pressor effect) and a negative sign indicates a decrease in blood pressure (depressor effect).

Compounds 12–20 appeared to possess central nervous system stimulatory properties, which precluded testing in dosages higher than those reported in Table II. Marked salivation was noted with N-(3-acetoxy-butyl)-O-acetyl ephedrine and N-acetonylmethyl-O-acetyl ephedrine (compounds 17 and 18) in the dose levels reported in Table II.

When sufficient material allowed, the compounds were screened to determine if any peripheral effects occurred at or below the dose levels employed for the analgetic tests. Table III summarizes the results of the studies which were carried out on isolated rabbit ileum strips and the circulatory systems of anesthetized cats.

Thirteen compounds were tested on the isolated rabbit ileum. Compound 1 had no distinct action. Compound 3 could be identified as a cholinergic stimulant of about 1/1,000 the potency of acetylcholine (Fig. 1). The spasmogenic effects of equivalent doses of each were blocked by atropine sulfate. The remaining 11 showed a generalized spasmolytic action by markedly inhibiting or

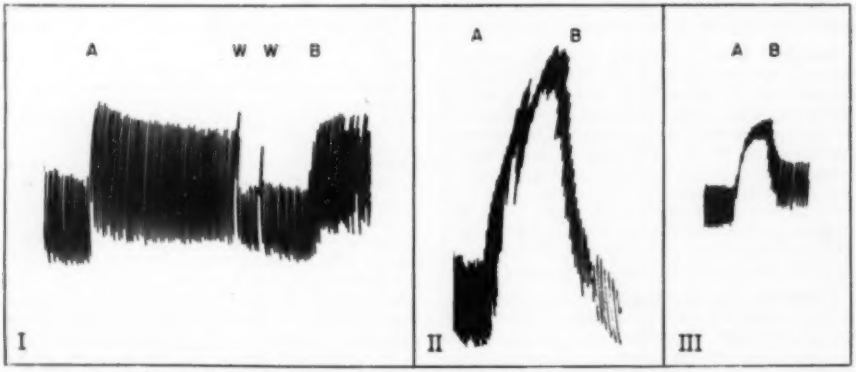


Fig. 1.—Effect of representative compounds on the isolated rabbit ileum.
I, left: A—acetylcholine 1:10,000,000; W—wash; B—compound 4 1:10,000.
II, center: A—barium chloride 1:100,000; B—compound 13 1:10,000.
III, right: A—acetylcholine 1:1,000,000; B—compound 13 1:10,000.

abolishing the spasm produced by acetylcholine and the increased activity produced by barium chloride. Typical spasmolytic effects are illustrated in Fig. 1.

Five compounds were tested for their effects on the blood pressure of the anesthetized intact cat as a reflection of any circulatory changes that they might produce. Compound 1 demonstrated no action of its own on the blood pressure. The cholinergic properties of compound 4 demonstrated on the intact rabbit ileum were verified by the ability of atropine sulfate to block its depressor action. Compounds 3 and 7 were depressor substances that did not modify the pressor effects of epinephrine and the depressor effects of acetylcholine. A typical depressor effect is illustrated in Fig. 2. Compound 1 demonstrated a more complex action producing an initial depression of blood pressure followed by a secondary rise. This is illustrated in Fig. 3.

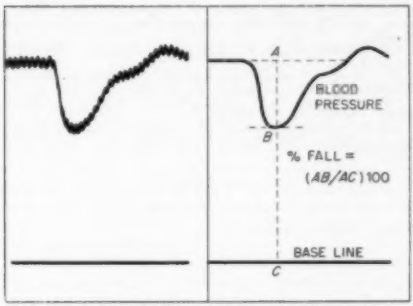


Fig. 2.—Typical depressor effect on blood pressure.
Left: Depressor effect produced by 1 mg./Kg. of compound 4.
Right: Method of calculating the magnitude of change in blood pressure.

It will be noted that all 5 compounds tested for their effect on the circulatory system of the cat showed analgetic activity with the D'Amour and Smith technique in the rat. From a comparison of the doses required to produce these pharmacologic effects, it would appear that, barring a distinct species variation, marked circulatory changes would be expected to occur at dose levels utilized to demonstrate the analgetic potency of these 5 compounds. Although the cholinergic nature of one of the compounds tested has been identified (compound 4), this cannot be taken as presumptive evidence that any of the other analgetic agents possess any autonomic properties or that the autonomic nervous system is involved in the analgetic mechanism.

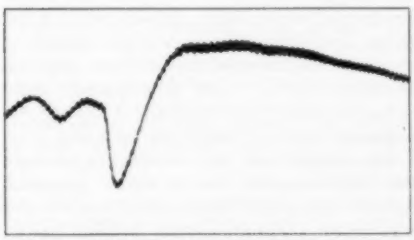


Fig. 3.—Effect of compound 1 on blood pressure.
Complex effect produced by compound 1 (8 mg./Kg.) on the blood pressure.

Of the 21 new compounds (Table 1) prepared to test the working hypothesis that the analgetic action of the morphine-type agent may be traceable to the presence of both sympathomimetic and parasympathomimetic moieties in the same molecule, twenty contain the aforementioned moieties and, according to the hypothesis, might be expected to possess analgetic activity. Of these 21, some degree of analgetic activity was demonstrated by 7, the yield of active compounds on the basis of the hypothesis being better than 33%.

It must be emphasized that the formulation of working hypotheses concerning analgetic activity are based upon the following unproved (although at this time seemingly plausible) contention: within the limits of our methods of observation, it has been tentatively concluded that the mechanisms of analgetic action of the so-called morphine-type analgetics are similar.

The results of this investigation permit an evaluation of the working hypothesis in regard to the necessary and sufficient requirements for analgetic action. It has already been noted (3) that all the active morphine-type analgetics contain within their structures some molecular aggregates that are structurally similar to present concepts of the sympathomimetic and parasympathomimetic moieties. There are also compounds that possess these moieties within their structures and are not active. Of the compounds prepared to test the working hypothesis, it should be noted that all conform to the hypothesis. One third of these show some degree of analgetic activity. Therefore, on the bases of the literature survey and this investigation, the aforementioned moieties, while not in themselves always sufficient for analgetic activity, are a necessary chemical constitutional requirement for this pharmacologic action. Thus the working hypothesis of Schueler, Gross, and Holland may be restated as follows: the presence of both a sympathomimetic and a parasympathomimetic moiety within the same molecule is an expression of the necessary chemical constitutional requirements for the morphine-type analgetics, although these moieties in themselves may not always be sufficient to confer analgetic action on the molecule. From a more practical standpoint, the results of this study indicate the usefulness of the working hypothesis as a means of approaching the synthesis of new agents of this pharmacologic class. A criterion for judging the relative usefulness of the hypothesis would be the question: what is the probability that 7 out of 21 compounds selected at random from the literature would possess analgetic activity?

While there have been many studies on the relationship between chemical constitution and analgetic action resulting from modifications in the structure of numerous type compounds, there has as yet been no attempt to evaluate the interaction of various portions of the molecule on analgetic activity. The necessity for this has already been intimated by Lee (1), and is emphasized by the results of this study.

This contribution is a continuation of the investigation of Schueler, *et al.* (3), and Slomka and Schueler (4).

SUMMARY

1. Ten new compounds were prepared and their analgetic potency has been determined on rats.

2. Thirteen of the compounds prepared in this investigation (Table I) were screened on the isolated rabbit ileum, and the effect of 5 of these on the circulatory system of the anesthetized intact cat were determined.

3. The results of this investigation illustrate the usefulness of working hypotheses based upon the considerations of the relationships between chemical constitution and pharmacologic action.

4. The results of this investigation warrant a restatement of the working hypothesis of Schueler, Gross, and Holland as follows: the presence of both a sympathomimetic moiety and a parasympathomimetic moiety within the same molecule is an expression of the necessary chemical constitutional requirements for the morphine-type analgetics, although these moieties in themselves may not always be sufficient to confer analgetic action on the molecule.

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The Bioassay of Some Commercial Conjugated Estrogens*,†

By WILLIAM HOMER LAWRENCE‡ and C. W. CHAPMAN§

Some commercial conjugated estrogens were assayed by oral (intragastric) administration to adult ovariectomized albino rats. The standard of reference used was 2-aminopyridine estrone sulfate. The products tested assayed from 46.4 per cent to 116.7 per cent of labeled potency. Only two manufacturers' products assayed up to the potency claimed on the label by this method. The bioassay procedure used in this study is suggested as a method of standardizing conjugated estrogens intended for oral therapy.

ALTHOUGH the majority of estrogenic materials currently used in therapy are pure crystalline substances not requiring biological standardization, certain noncrystalline preparations derived from pregnant mare's urine are standardized by biological procedures (1). These preparations, namely, conjugated estrogens or estrogenic substances (water-soluble), are widely used and in recent years have been made available by a number of different manufacturers.

Because there is no official assay method nor reference standard for conjugated estrogens, the study reported herein was undertaken in order to evaluate biologically some of the conjugated estrogen products available commercially. Since these water-soluble products are intended for oral administration, it was decided to parallel the clinical use by a bioassay method in order to determine the relative oral efficacies of the commercial preparations. A modification of the Kahnt and Doisy (2) test was the method of assay: graded doses of estrogens and reference standard material were administered orally (intragastrically) to ovariectomized rats and the responses thereby induced were compared.

The material used as a standard of reference was 2-aminopyridine estrone sulfate (hereafter referred to as APES) which is a more stable and manageable preparation than sodium estrone sulfate (SES), one of the principal estrogens present in the conjugated mixed estrogens.

* Received July 19, 1952, from the School of Pharmacy, University of Maryland, Baltimore.
† Abstracted from a thesis submitted to the Graduate School of the University of Maryland by William Homer Lawrence in partial fulfillment of the requirements for the degree of Master of Science.
‡ Grateful acknowledgment is made to Dr. C. T. Ichniowski for advice in the preparation of this manuscript and to Miss Georgianna S. Gittinger for technical assistance.
§ Deceased April 5, 1951.

EXPERIMENTAL

Preparation of Assay Material.—The conjugated estrogens studied were the products of eight different manufacturers. Samples were purchased on the open market. They were of two strengths and were labeled as containing either 1.25 mg. or 0.625 mg. of conjugated estrogens expressed as sodium estrone sulfate.

The tablets were crushed and suspended in 10% ethanol. From this suspension the proper dilutions were prepared to give the required graded dose levels for the assay when 1 cc. per rat of the proper dilution was administered daily for three consecutive days. A stock solution of APES was prepared by dissolving an accurately weighed quantity of APES in sufficient alcohol (95% ethanol) to give 1 mg./ml. The graded dose dilutions were prepared from a buffered (*p*H 7.0) 1:10 dilution of this stock solution.

Priming.—Before any animals were used for assay purposes, they were given intraperitoneal injections of 10 μg. of APES and vaginal smears were taken to ensure that all animals were capable of responding to administered estrogens. The animals not giving positive responses after the first priming dose were considered suitable assay animals provided that they gave a positive response after a second priming dose.

The assays were conducted on alternate weeks. The rats were kept responsive by priming on Mondays of the intervening weeks. If for any reason rats were not used for an assay in a designated week, they were kept in condition by a priming dose for that week also. This precaution was taken to preserve suitability and responsiveness among the test animals.

Assay Procedure.—A group of 20 animals was used for each dose level of reference material and assay sample administered, with the exception of one assay in which 15 rats per dose level were used. Three doses of standard and assay sample, respectively, were administered to obtain responses used in the estimation of potency after the effective doses of the materials had been determined by orientation assays.

The doses were administered to the rats by means of a 5-cc. syringe fitted with a suitable length of No. 8 rubber catheter so that the dose was delivered directly into the stomach of the animal. Administration was made daily for three consecutive days. On the fourth and fifth days, vaginal smears were obtained twice daily (9 a. m. and 3 p. m.) and were examined (unstained) microscopically (100×) to determine the degree of cornification produced.

Smearing and Interpretation of Smears.—The vaginal smears were obtained by flushing the vagina of the rat with approximately 0.2 to 0.25 cc. of saline, using a suitable medicine dropper, and withdrawing a part of the cell suspension. This suspension, placed on a plain microscope slide with no cover glass, was examined while still wet under 100× magnification. Those smears showing predominantly squamous cells (cornified epithelial cells) were considered positive (+) and given a numerical value of one; those containing a majority of nucleated epithelial cells with only a minority of squamous cells or leukocytes were considered partial (±) and given a value of one-half; and those exhibiting predominantly leukocytes were considered negative (−) and given a value of zero. This scoring method was used to calculate per cent response on all dose levels. The per cent response was converted to probits by consulting per cent-probit tables (3).

The relationship between the estrogenic activity of the standard and that of the tablets being assayed was determined graphically by plotting the probit on the ordinate and the dosage on the logarithmic scale as the abscissa on two-cycle, semi-logarithmic paper. The RD_{50} of the standard, in mg. of APES, and that of the assay sample, in fractions of a tablet, were obtained and equated so as to express the tablet potency in terms of mg. of APES. The theoretical relationship between APES and SES on a molecular weight basis is 1:0.84. This equivalence was confirmed by bioassay. The biological potency of the tablets was converted to SES equivalence by multiplying the APES value obtained by the conversion factor 0.84. The SES equivalences of the tablets established by bioassay were compared with the label statements. The assayed estrogenic content of each product then was expressed as per cent of labeled content.

The results obtained are given in Tables I and II. In Table I the results were obtained by direct graphic analyses whereas the results in Table II were obtained by calculations according to the method of Irwin (4).

Results obtained from two assays of APES against SES showed the following equivalences: 1:0.8525 and 1:0.8066. These bioassay results are in accord with the theoretical relationship of 1:0.84.

TABLE I

Product	Assay Results: —% of Label-Stated Contents—				Average, %
A	67	88.4	75.9	73.7	76.25
B	57.1	42.4	54.2	...	51.2
C	112	100.8	125.6	...	113.1
D	83.2	...	...	...	83.2
E	119.2	114.2	...	...	116.7
F	58.5	76.4	88.2	...	74.4
G	76.2	65	65.8	...	69.0
H	46.4	...	...	...	46.4

DISCUSSION

It is generally believed that most estrogenic products are inactivated in the liver (5–7), presumably by an oxidative enzyme, inasmuch as this inactivation is inhibited by NaCN (8). Since a substance that is absorbed from the gastrointestinal tract must pass through the liver as it enters the circulation to reach the target organs, except in special cases of oily substances that may by-pass the liver via the thoracic duct (9), a modified Kahnt and Doisy test was used for assay in order to parallel, as nearly as possible, the actual therapeutic usage of this type of drug. This method of assay is used in order to subject the drugs to as nearly the same physiologic conditions during the course of the assay as the products meet during therapy. This type of method would tend to rule out the problem of determining the ratio of the various natural estrogens obtained during the extraction, since the

assay would yield an estimate of the total oral estrogenic activity.

At present there is no generally accepted method of standardizing conjugated estrogens. It is further recognized that there is difficulty with the present chemical methods of estimation (10, 11).

It is believed that the method of assay presented in this report would provide a reliable method in the standardization of conjugated estrogens intended for oral therapy.

Because of the instability and hygroscopic character of pure SES, another estrone preparation, APES, was used as the standard for comparison during the course of the assays. Grant and Beall (12) have demonstrated the ease and rapidity of decomposition of solutions of SES at 20 and 37° C. and at various acid *p*H levels.

TABLE II

Product	Assay Results: —% of Label-Stated Contents—				Average, %
A	68	86.4	75.2	74.4	76.0
B	57.6	42	53.4	...	51.0
C	113.6	103.2	122.4	...	113.1
D	86.4	...	...	...	86.4
E	123.2	118.4	...	...	120.8
F	60	76	87.2	...	74.4
G	76.8	65.6	69.6	...	70.7
H	47.2	...	...	...	47.2

CONCLUSIONS

1. At present there is no generally accepted method of standardizing conjugated estrogens nor is there an accepted reference standard.

2. There is a serious lack of uniformity in the oral bioassay potencies of representative samples of commercial conjugated estrogens marketed for oral administration. Among the eight brands assayed, potencies ranged from 46.4 per cent to 116.7 per cent of labeled claim when assayed by this method. Only two products, C (Conestron) and E (Premarin), possessed the estrogenic activity stated on the label when determined by this method.

3. These findings indicate an urgent need for the adoption of a standard assay method and reference standard for conjugated estrogens.

4. The method of assay used in this study is proposed as a reliable method, since such a procedure of oral administration reflects clinical usage of the preparations tested.

5. Aminopyridine estrone sulfate serves as a very satisfactory reference standard since it is qualitatively similar and has the advantage over sodium estrone sulfate of being nonhygroscopic and stable for ordinary procedures.

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BOOKS RECEIVED

Nutrition in the Practice of Medicine. Nutrition Symposium Series, No. 4. The National Vitamin Foundation, Inc., New York, 1952. ii + 163 pp. 15.5 x 23 cm. Price $1.50.

Pharmaceutical Calculations. 2nd ed. By WILLIS T. BRADLEY, CARROLL B. GUSTAFSON, and MITCHELL J. STOKLOSA. Lee & Febiger, Philadelphia, 1952. 290 pp. 20.5 x 14 cm. Price $3.75.

Substances Naturelles de Synthese. Vol. IV. By DR. LEON VELLUZ. Masson et Cie., Paris, 1952. 165 pp. 16 x 23.5 cm. Price 1500 fr.

Bacitracin. Research Division, S. B. Penick & Co., New York, 1952. 127 pp. 14.5 x 22 cm. Illustrated.

Applied Physiology. By SAMSON WRIGHT. Oxford University Press, New York, 1952. xvi + 1190 pp. 22.5 x 15 cm. Illustrated. Price $9.00.

Methods in Medical Research. Vol. 5. A. C. CORCORAN, Editor-in-Chief. The Year Book Publishers, Inc., 1952. xiii + 394 pp. 14.5 x 22.5 cm. Illustrated. Price $7.50.

An Introduction to the Chemistry of the Hydrides. By DALLAS T. HURD. John Wiley & Sons, Inc., New York, 1952. x + 231 pp. 23.5 x 15.5 cm. Price $5.50.

Chemistry of Carbon Compounds. Vol. I, Part B. Edited by E. H. RODD. Elsevier Press, Houston, Texas, 1952. xvi + 1462 pp. 16.5 x 23.5 cm. Price $17.50 (single copy); $15.00 (subscription).

Book Notices

Methods in Medical Research. Vol. 5. A. C. CORCORAN, Editor-in-Chief. The Year Book Publishers, Inc., 1952. xiii + 394 pp. 14.5 x 22.5 cm. Illustrated. Price $7.50.

While this book is primarily of medical application, it also includes much of pharmaceutical interest. Many of the methods described can be adapted to the needs of research workers not directly concerned with problems related to basic medical research. The book is divided into three sections. The first section, dealing with methods for separation of complex mixtures and higher molecular weight substances, is of a more general nature. In this section, applications involving countercurrent distribution, paper chromatography, electrophoresis, and ultracentrifugation are discussed. In section II, methods of renal study are covered by ten contributions, several of which are divided into two or more parts. This section includes much of pharmacological and biochemical interest. Section III, devoted to immunochemical methods for determining homogeneity of proteins and polysaccharides, includes immunological methods of a wide scope and importance based on information not otherwise readily accessible.

Applied Physiology. By SAMSON WRIGHT. Oxford University Press, New York, 1952. xvi + 1190 pp. 22.5 x 15 cm. Illustrated. Price $9.00.

This comprehensive textbook on physiology has now been revised eight times since the first edition was published in 1926. This record indicates, to some extent, the popularity of the work. The new ninth edition of *Applied Physiology* has undergone a more thorough revision than any heretofore. The subject matter has been rearranged and more than half the text has been completely rewritten to reflect recent advances in physiology. The number of illustrations has been increased by the inclusion of 200 new figures chosen mainly from recent papers. Throughout the book the clinical aspects of physiology are stressed, but despite this emphasis it should serve as a useful reference book for students whose interests are more inclined toward a general treatise on physiology and those who expect to become physicians. The book is documented with references to review articles and other papers pertinent to each subject discussed. The volume is provided with an unusually complete index, and the printing and binding are of good quality.

Probit Analysis. 2nd ed. By D. J. FINNEY. Cambridge University Press, New York, 1952. 22.5 x 14 cm. xiv + 318 pp.

As indicated by the sub-title, "A Statistical Treatment of the Sigmoid Response Curve," this book deals with the application of statistics to biological assays. These assays are used to determine the effect and efficiency of insecticides, fungicides, drugs, vitamins, and other substances.

The text has been prepared in an effort to standardize the methods of probit analysis and to make its use available to the biologist without statistical training. The main outline of the fundamental mathematical theory is indicated. The theory of the analysis of quantal response data is discussed.

The text material is covered under the following headings: Introductory (Definitions and Terms), Quantal Responses and the Dosage-Response Curve, Median Effective Dose, Maximum Likelihood Solution, Comparison of Effectiveness, Adjustments for Natural Mortality, Factorial Experiments, Toxic Action of Mixtures of Poisions, Graded Responses, Miscellaneous Problems, and Recent Developments.

The author's method of illustration by example is developed with the use of 34 examples covering as many different problems. The large number of examples is indicative of the rapid growth in this field since the appearance of R. A. Fischer's *Statistical Methods for Research Workers* in 1925. The explanations in Dr. Finney's book are clear, but one feels that unless the worker is constantly in touch with this subject, the advice that one should consult a statistician in these studies is more pertienent now than when Fisher offered it in 1925.

This book can be helpful to the novice and to the expert in the bioassay field, and it should be available in reference libraries.

Experimental Nucleonics. By ERNST BLEULER and GEORGE J. GOLDSMITH. Rinehart and Company, Inc., New York, 1952. xv + 393 pp. 16 x 23.5 cm. Price $6.50.

This book has been designed to serve as a basis for the training of people planning to perform tracer research, as well as for students of nuclear physics. It is not an elementary textbook, and in order to read it with understanding, a background of the fundamentals of nuclear physics and of chemistry is necessary.

The book is conveniently divided into four major sections. In the first division is presented information concerning the laboratory itself, the equipment, procedures in health protection, and other essential background material. Under the division headed "General Techniques," operations essential to anyone working in the field, regardless of the specialty involved, are described. This section includes many counting experiments involving equipment that will ordinarily be encountered in experimental work on nuclear radiations. Throughout the section the role of chemistry is emphasized. In the other two sections chemical techniques and physical techniques, including numerous experiments, are adequately treated.

The book is documented with references for supplementary reading and is provided with an appendix containing useful physical constants and with author and subject indexes. This volume should be useful to any worker in the field of experimental nucleonics.

Ultraviolet Radiation. By LEWIS R. KOLLER. John Wiley and Sons, Inc., New York, 1952. ix + 270 pp. 15.5 x 24 cm. Illustrated. Price $6.50.

This book is designed to furnish basic information which may be required by the nonspecialist in the field of radiation. It will also serve to answer some of the questions which confront physicists as well as specialists in fields other than physics when they find it necessary to work in this portion of the spectrum. The volume can be used to advantage by those in the pharmaceutical field, and even by laymen, as a source of concise and authoritative information on certain phases of ultraviolet radiation.

The book consists of eight chapters, the first of which furnishes an introduction to the subject including basic definitions, physical units, kinds of spectra, and (of particular interest in the pharmaceutical field) explanations of biological units employed in the field. In the second chapter several commercial arcs are described in order to illustrate certain types of discharges. Next are presented chapters on reflection and transmission restricted to materials which are most commonly used and readily available. The seventh chapter, which is devoted to applications and effects of ultraviolet radiation, discusses erythema, the application of ultraviolet radiation in the prevention and cure of rickets, the production of Vitamin D, germicidal effects, and the disinfection of air, water and other liquids. In this chapter the fluorescent lamp is also adequately described. In the eighth chapter, physical, chemical, and biological detectors of ultraviolet radiation are discussed, with particular emphasis on the physical detectors which are the most important group.

The author, Dr. Lewis R. Koller, who is a Research Associate in the General Electric Research Laboratory in Schenectady, New York, has succeeded in writing a most interesting and understandable book on a subject not too easily comprehended by a nonspecialist in this field. He has apparently more than realized his aim in writing the book. The value of the volume is enhanced by pertinent references at the end of each chapter and by a well designed index. The printing, binding, and typography are attractive and of good quality.

Glycols. Edited by GEORGE O. CURME, JR., and FRANKLIN JOHNSTON. Reinhold Publishing Corp. New York, 1952. xii + 389 pp. 16 x 23.5 cm. Price $12.00.

This book represents another important addition to *The American Chemical Society Monograph Series.* The editors and contributing authors are research-staff members of the Carbide and Carbon Chemical Corporation, and have succeeded admirably in compiling complete information on all glycols now in production and those which can be produced on a commercial scale. The subject matter is presented mainly in terms of ethylene glycol and propylene glycol. Their production, physical properties, applications, oxides, derivatives, and condensation polymers are covered in separate chapters, and a portion of the book is devoted to higher glycols, toxicological data, and analytical methods.

Manufacturing techniques including details of two commercial methods for producing ethylene oxide from ethylene chlorohydrin and by direct oxidation of ethylene are described. Because of the application of the glycols in the pharmaceutical and cosmetic fields and the potential that exists for expanding their fields of application, this book should be of especial interest to research workers in these areas. The book is well documented, and is supplied with an author index and a complete subject index. The binding and printing are of the same style and quality used in other members of *The American Chemical Society Monograph Series.*

Pharmacopoeia Japonica VI. Japanese Pharmaceutical Association, Tokyo, Japan, 1951. 756 pp. 15 x 21.5 cm.

The sixth edition of the *Pharmacopoeia of Japan (1951)* is the first complete revision of that compendium since 1932. The changes in materia medica during that period were many, and the latest revision was prepared by the Subcommittee on Official Compendium of the Pharmaceutical Affairs Committee as authorized by the Minister of Welfare in 1947, using the U. S. P. XIII as a guide. The English translation, published in 1952, is an excellent book of standards.

Aside from minor variations, the monograph style is similar to that of the U. S. P. As in the P. I., single and daily usual and maximal doses are given. The main differences arise from language variations, official nomenclature, and use of Latin for the main official titles. Most of the Latin and English titles have the similarity that is noted in the U. S. P.; however, some deviations from this form are noted, which tend to make the student's task more difficult, as in the following Latin-English title combinations: Chinoformum-Iodochlorohydroxyquinoline, Chinoiodinum-Chiniofon, Sulfaminum-Sulfanilamide, Epirenamina-Epinephrine.

The titles for Hexobarbital (N. N. R.) is given as Methylhexabitalum and Methylhexabital. This nomenclature does not indicate the chemical and therapeutic identity of this compound with the barbituric acid derivatives. Aminopyrine is included, and so is a 2:1 mole combination of aminopyrine and barbital. An analogous mixture containing amobarbital is available in the United States.

When an element or a compound other than the title material appears in the rubric statement of a monograph, the atomic or molecular weight is given. This is a helpful reference aid, especially for analytical calculations.

The arrangement of the J. P. VI (official abbreviation) makes it a convenient reference volume, especially for comparative checking with other drug compendia. The similarity of the contents of recently published official compendia, including the P. I., make one sharply aware that in the field of health the geographical fences are disappearing.

Useful Drugs. 15th ed. J. B. Lippincott Co., Philadelphia, 1952. xi + 262 pp. 19.5 x 13 cm. Price $2.50.

This book is published by the Council of the American Medical Association to encourage the practice of rational therapeutics. The Council has selected from the U. S. P. XIV, N. F. IX, and N. N. R. 1950, those drugs of recognized merit in the opinion of the Council members.

The text is a selected materia-medica containing information on the chemical and physical properties, as well as the actions, uses, and dosages of the included drugs. Tables of weights and equivalents and definitions of types of pharmaceutic preparations are included. A therapeutic index, a classification of drugs by diseases for which they are used, and a general index are appended.

The Council recommends the use of this pocket-size volume as a basis for medical school instruction and state board of medicine examinations.

Bacitracin. Research Division, S. B. Penick and Co., New York, 1952. 127 pp. 14.5 x 22 cm. Illustrated.

This book was designed to make available a review of the data on the clinical usefulness of bacitracin which have accumulated since its discovery in 1945. It serves to direct thought to clinical applications of bacitracin which have been employed thus far and to suggest its potentialities through its known synergistic action with other antibiotics. The book is divided into ten chapters in which the history, chemistry, methods of assay, pharmacology, bacteriostatic aspects, clinical applications, synergistic action, and dosage forms are discussed. In one chapter the applications of bacitracin in veterinary medicine are recorded; the final chapter consists of a summary of the properties of this important antibiotic.

The Pharmaceutical Curriculum. By LLOYD E. BLAUCH and GEORGE L. WEBSTER. American Council on Education, Washington, D. C. Printed by George Banta Publishing Co., Mensaha, Wisconsin, 1952. xvi + 257 pp. 16 x 23.5 cm. Price $2.00.

This report, prepared by the authors for the Committee on Curriculum of the American Association of Colleges of Pharmacy, is an important and tangible result of The Pharmaceutical Survey. In this book, the data and concepts upon which the recommendations of the Committee on The Pharmaceutical Survey relating to the general and professional education of the pharmacist were based have been expanded and presented in a logical and orderly style.

The book consists of thirteen chapters, including an introduction and a summary of the principal conclusions reached from the pharmaceutical curriculum studies. The second chapter outlines briefly but clearly the development of pharmaceutical education in the United States between 1900 and 1950. Thus, a background for the main portion of the report is supplied. In chapter 3 the objectives to be accomplished by a program in pharmaceutical education are explored and explained. The following chapter deals with facts and ideas concerning the place of general education in the pharamaceutical curriculum. In the next five chapters, the several fields and divisions of pharmaceutical education are thoroughly discussed. Instruction suggested in the various subjects is characterized and described, and in some instances quite detailed outlines of suggested content of certain courses are given. The relationship between the number of hours spent in classes and in the laboratory to units of credit is thoroughly explored, and definite recommendations are made. Chapters 10, 11, and 12 are concerned with the curriculum as a whole, and one of these chapters outlines certain interesting general principles for curriculum improvement. The book is provided with an excellent index which facilitates its use.

The Pharmaceutical Curriculum is competently written and its design and arrangement are excellent. It is an outstanding contribution to pharmaceutical education and will undoubtedly accomplish much in its development and improvement in the years immediately ahead. Its careful reading should be required by all individuals concerned with the professional education of the pharmacist. It should also be read, and its implications carefully considered, by those representing the several phases of pharmaceutical activity that will require the services of graduates in pharmacy in the future.

Diseases in Old Age. By ROBERT T. MONROE. Harvard University Press, Cambridge, Mass., 1951. xi + 407 pp. 15 x 21.8 cm. Price $5.00.

This book presents a clinical, pathological, and therapeutic survey of the records of all patients over sixty-one years of age who have availed themselves of the facilities of the Medical Services of the Peter Bent Brigham Hospital in Boston, Mass., since March, 1913. The book presents a good argument for the establishment of old-age centers to promote the general welfare of the aging and the aged. Its primary interest is medical in nature, but it is written in a style that should serve to furnish valuable information to social service workers and others interested in geriatrics.

Chemistry of Carbon Compounds. Vol. I, Part B. Edited by E. H. RODD. Elsevier Press, Houston, Texas, 1952. xvi + 1462 pp. 16.5 x 23.5 cm. Price $17.50 (single copy); $15.00 (subscription).

Volume I, Part A, of this work was reviewed [THIS JOURNAL, **41**, 169(1952)], and the design and plans for subsequent volumes were described. Volume IB continues with the systematic treatment of aliphatic compounds which was started in Volume IA. It follows the same plan as that described previously, and includes an unusually complete treatment of compounds belonging in the carbohydrate series. Some organic chemists might question the advisability of including an account of the carbohydrates with the aliphatic compounds since members of this group exist largely in a cyclic form. The important feature of the chapter on hexa- and polyhydric alcohols and their oxidation products, by J. K. N. Jones of Bristol, however, is the evident excellence and completeness with which the chemistry of the carbohydrates and their derivatives have been presented. Other important chapters include proteins and enzymes; these portions appear to be entirely modern in their approach to the discussion of the constitution of these biologically important substances. Judging from Volume I, Parts A and B, the chemistry of the carbon compounds will serve very adequately as a successor to Richter's *Organic Chemistry* as a valuable reference book. Chemists in the United States interested in organic compounds who have had an opportunity to examine Volume I will look forward

with keen anticipation and interest to the publication of subsequent volumes planned to complete the series.

Volume II will be devoted to the alicyclic compounds and will include information on rubber and rubberlike high molecular substances because of their relation to the terpenes. The aromatic compounds, heterocyclic compounds, and miscellaneous compounds will be dealt with in Volumes III, IV, and V, respectively.

Pharmacognosy of Indian Leaf Drugs. By S. C. DATTA and B. MUKERJI. Pharmacognosy Laboratory, Bulletin No. 2, Ministry of Health, Government of India. Government of India Press, Calcutta, India, 1952. iv + 124 pp. 18 x 26 cm.

This book represents a continuation of a study on the pharmacognosy of drugs indigenous to India. It is the second in a series of which the first was devoted entirely to Indian root and rhizone drugs. In this volume only leaf drugs are considered, and these include both well-known leaf drugs and those employed by the Ayurvedic practitioners of medicine in India. Each monograph includes a description of pharmacognostic characteristics and a brief account of the therapeutic uses of each drug in modern as well as in the indigenous system of Indian medicine. The volume is profusely illustrated with drawings of the principal macroscopic and microscopic characteristics of the Indian drugs by which they may be identified. The book should be of interest to pharmacognosists and perhaps to others who might be interested in leads for investigations of drugs used in Ayurvedic medicine, but concerning the nature of which little is known at the present time.

Substances Naturelles de Synthese. Vol. IV. By LEON VELLUZ. Masson et Cie., Paris, 1952. 165 pp. 16 x 23.5 cm. Price 1500 fr.

This French volume was prepared by Dr. Velluz and the same collaborators who produced the earlier three volumes which were reviewed in THIS JOURNAL, **40,** 534(1951); **41,** 167(1952). The same arrangement and treatment of the subject matter is followed in this fourth volume as was reported for the earlier volumes.

Detailed methods of synthesis are given for D-pantothenic acid, adermine (pyridoxine), riboflavine, testosterone, and DL-α-tocopherol. Less detailed reviews are given for related compounds and intermediates.

A review of the methods, with references, used in the synthesis of compounds containing the pyridine nucleus is given. The formulas, reaction schemes, methods, and references are tabulated.

A brief review of chromatographic adsorption techniques, materials, and applications is presented, augmented by a tabulation of separated mixtures, including the adsorbent, solvents, and reference in each case. Several examples are given in detail.

These volumes are excellent short reviews of the text material.

A Laboratory Manual for Inorganic Pharmaceutical Products. By ROBERT F. DOERGE and CHARLES O. WILSON. Hemphill's, Austin, Texas, 1952. 196 pp. 23 x 27.5 cm.; and

A Study Manual for Inorganic Pharmaceutical Products. By Tony E. Jones and Charles O. Wilson. Hemphill's, Ausin, Texas, 1952. 144 pp. 23 x 27.5 cm.

This excellent laboratory manual is designed for undergraduate students in pharmacy who have had a basic course in inorganic chemistry, to acquaint them with the properties of organic compounds used as drugs. Experiments are presented dealing with the preparation of inorganic compounds and their physical and chemical properties. The emphasis on type reactions in the experiments outlined should provide valuable background for the student upon which he may later draw when problems involving incompatibilities arise in dispensing prescriptions, or when he may need to develop dosage forms of inorganic pharmaceutical chemicals alone or in combination with other drugs.

The study manual provides the student with a list of questions on the most important compounds and includes lists of available proprietary preparations in which each inorganic compound occurs. The questions are well designed, and by following the study manual the student is aided greatly in determining the intent of the experiments in the laboratory manual and the knowledge that he should gain therefrom.

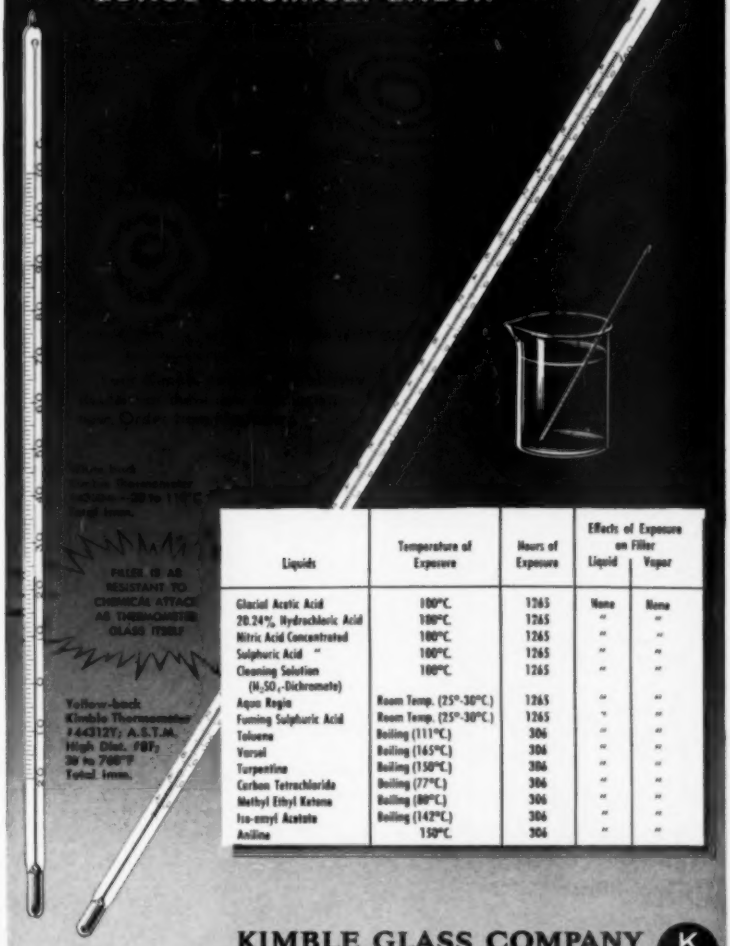
The chapters are grouped according to the arrangement used in the Fifth Edition of *Inorganic Pharmaceutical Chemistry,* by Rogers, Soine, and Wilson.

Nuttall's Travels into the Old Northwest: An Unpublished 1810 Diary. By JEANNETTE E. GRAUSTEIN. No. 1/2, Volume 14 of Chronica Botanica. The Chronica Botanica Co., Waltham, Mass.; Stechert-Hafner, Inc., New York; 1952. Illustrated. viii + 88 pp. 17.5 x 26 cm. Price $3.00.

It will be recalled that Thomas Nuttall was an English botanist who came to the United States about 1808 and conducted many botanical explorations upon results of which he based his *Genera of North American Plants* which was published in 1818. He was Professor of Natural History at Harvard University from 1822 to 1834. Many sketches of Thomas Nuttall's life have been published. This book adds to a knowledge of the character of the man and of his work and accomplishments. It consists of a heretofore unpublished diary covering a period between April and September, 1810, constituting a record of the first months of his expedition of that year. The story told in this diary is most interesting and its value to the historian and to the casual reader is enhanced by annotations by the editor of the diary. The diary is preceded by an excellent introduction outlining the route followed, the hazards involved, and a chronological account of Nuttall's travels into the Old Northwest.

TESTS PROVE...

Permanent Filler in Kimble Thermometers defies chemical attack


FILLER IS AS RESISTANT TO CHEMICAL ATTACK AS THERMOMETER GLASS ITSELF
Yellow-back Kimble Thermometer #44312T; A.S.T.M. High Dist. 98F; 39 to 768°F Total Imm.

Liquids	Temperature of Exposure	Hours of Exposure	Effects of Exposure on Filler: Liquid	Vapor
Glacial Acetic Acid	100°C.	1265	None	None
20.24% Hydrochloric Acid	100°C.	1265	"	"
Nitric Acid Concentrated	100°C.	1265	"	"
Sulphuric Acid "	100°C.	1265	"	"
Cleaning Solution (H_2SO_4-Dichromate)	100°C.	1265	"	"
Aqua Regia	Room Temp. (25°-30°C.)	1265	"	"
Fuming Sulphuric Acid	Room Temp. (25°-30°C.)	1265	"	"
Toluene	Boiling (111°C.)	306	"	"
Varsol	Boiling (165°C.)	306	"	"
Turpentine	Boiling (150°C.)	306	"	"
Carbon Tetrachloride	Boiling (77°C.)	306	"	"
Methyl Ethyl Ketone	Boiling (80°C.)	306	"	"
Iso-amyl Acetate	Boiling (142°C.)	306	"	"
Aniline	150°C.	306	"	"

KIMBLE GLASS COMPANY

Toledo 1, Ohio

Subsidiary of Owens-Illinois Glass Company

Baker provides increased capacity...

more AMMONIUM SULFATE

to meet the requirements of exacting users

Our ability to provide measured purity within a narrow range of tolerances has continually increased the demand for Baker's Ammonium Sulfate, particularly for syringeable products.

To meet the increased demand for this important chemical, the J. T. Baker Chemical Co. has enlarged its capacity with expanded production facilities. An additional plant with new stainless steel equipment of the latest design now makes available a more abundant supply of Ammonium Sulfate of extreme purity—assay 99.5% minimum $(NH_4)_2SO_4$—low in heavy metals content—of controlled pH—with low chloride content—factors of particular importance to manufacturers of biologicals and antibiotics.

When it is essential to keep heavy metals at a very minimum—when *measured purity* and *constant uniformity* mean less production difficulty—when your good name and the reputation of your product depend on your choice of vital ingredients—specify Baker's Ammonium Sulfate.

Baker's years of "know how" and Baker's added manufacturing facilities enable us to give service to additional manufacturers who require quality Ammonium Sulfate.

For further information and samples of Ammonium Sulfate or any of the long list of Fine Chemicals Baker has available, write Fine Chemical Division, J. T. Baker Chemical Co., Phillipsburg, New Jersey.

Test Baker and *COMPARE!*

$(NH_4)_2SO_4$

SPECIFICATIONS

PURIFIED GRANULAR

F. W. 132.146

Assay $(NH_4)_2SO_4$	99.5%
Insoluble Matter	0.010%
Residue After Ignition	0.015%
Neutrality	Passes Test
Chloride (Cl)	0.005%
Arsenic (As)	0.0005%
Heavy Metals (as Pb)	0.002%
Iron (Fe)	0.001%

Baker CHEMICAL

Baker Chemicals

REAGENT • FINE • INDUSTRIAL